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IDENTIFICATION AND CHARACTERIZATION OF NOVEL GENETIC AND EPIGENETIC FACTORS REQUIRED FOR NORMAL AND MALIGNANT HEMATOPOIESIS

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Identification and characterization of novel genetic and epigenetic factors required for normal and malignant hematopoiesis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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The desire for science I could not forego,
Few secrets remained that I did not know
Seventy-two years, day and night I thought
Yet I came to know, I have nothing to show
Omar Khayyam (1048-1131)

To Scientists,
who have always devoted their lives to a better world

ABSTRACT

Acute myeloid leukemia (AML) is a type of blood cancer, characterized by clonal expansion and loss of differentiation ability of myeloid progenitor cells leading to abnormal accumulation of immature myeloid cells (myeloblasts) in the bone marrow and peripheral blood.

This thesis (study I to IV) focused on the identification and characterization of genes which are required for AML growth. The final study (study V) aimed to uncover the role of *NAP1L3* in normal hematopoietic stem cells (HSCs).

In studies I and II, we performed large-scale RNA interference screens in mouse and AML human cell lines to identify novel factors and pathways required for AML growth. Using this approach, we identified two novel targets: Chromatin remodeling factor *CHD4* (study I) and the transcription factor *GTF2IRD1* (study II), which display both a strong inhibitory effect on the growth of AML cells and a less negative effect on normal hematopoietic cells.

Using RNA interference and CRISPR-Cas9 techniques, we revealed that these genes were crucial for AML cell growth *in vitro* and *in vivo*. Knockdown of either *CHD4* or *GTF2IRD1* accumulated cells in the G0 phase of the cell cycle and resulted in downregulation of *MYC* and its target genes. We demonstrated the inhibitory role of *CHD4* knockdown on the growth and maintenance of primary childhood AML in an *ex vivo* setting, as well as in a xenograft model by transplanting patient-derived samples into humanized NSG-SGM3 mice. *GTF2IRD1* knockdown reduced the number of primary childhood and adult AML cells in *ex vivo* culture and delayed AML progression in the transplanted animal model. Therefore, *CHD4* and *GTF2IRD1* are important for AML cell growth, and interestingly the knockdown of these two genes did not show a strong inhibitory effect on normal hematopoietic cell growth.

In study III, we described the role of an epigenetic enzyme, the histone methyl-transferase *EHMT1* in AML. We used RNA interference, CRISPR-Cas9, and pharmacological approaches to inhibit *EHMT1* expression, which prevented the growth of various AML cell lines and primary AML patient samples. Knockdown of *EHMT1* significantly delayed disease progression in AML mouse models and prolonged their survival. Next, we employed CRISPR-Cas9 technology to generate single and double gene knockouts of *EHMT1* and its homolog *EHMT2*, which showed that both enzymes cooperatively play a role in AML cell proliferation and shared a similar cellular mechanism as individual knockouts of either gene resulted in an increased number of cells in G0 phase of the cell cycle. RNA sequencing of

the transcriptome of AML cells with *EHMT1* and *EHMT2* knockdown identified several common biological processes, including cell differentiation, proliferation and survival, as well as other unshared pathways and downstream effectors.

In study IV, we contributed to Nikolas Herold's study, who found that deoxynucleoside triphosphate (dNTP) triphosphohydrolase SAM domain and HD domain 1 (*SAMHD1*) plays a role in detoxifying intracellular ara-CTP in cells treated with the deoxycytidine analog cytarabine (ara-C). Transient reduction of SAMHD1 expression by using the simian immunodeficiency virus (SIV) protein Vpx significantly increased the sensitivity of AML cells to ara-C, whereas AML cells lacking *SAMHD1* transplanted into recipient mice were hypersensitive to ara-C. We showed that *in vitro* treatment of primary AML patient samples with Vpx, which suppresses SAMHD1, resulted in reduced proliferation of AML but not normal cells. Together, our data suggest that SAMHD1 inhibition can be used as a therapeutic strategy for cancer (AML) patients with high SAMHD1 expression.

In study V, our aim was to identify novel epigenetic regulators of normal HSCs. We found high expression level of *Nap1l3*, a member of nucleosome assembly proteins (NAPs), as a histone chaperone in HSCs. Loss of function of mouse *Nap1l3* mediated by shRNA or CRISPR-Cas9 impaired the maintenance and differentiation of HSCs in both our *in vitro* and *in vivo* studies. Moreover, downregulation of *NAP1L3* in human UCB HSCs significantly decreased both the number of colonies formed by HSCs and their proliferation *in vitro* due to cell cycle arrest in the G0 phase. Xenograft mouse models using human HSCs with *NAP1L3* knockdown showed a reduction of HSC reconstitution and bias in differentiation. Furthermore, we observed upregulation of several *HOX* genes (*HOXA3*, *HOXA5*, *HOXA6* and *HOXA9*) under *NAP1L3* suppression in human HSCs.

Altogether, in this thesis, we showed the important roles of *CHD4*, *EHMT1* and *GTF2IRD1* in AML cell expansion, identifying them as potential novel targets for AML treatment. Moreover, we revealed the cellular mechanisms and RNA expression patterns under knockdown of these genes. We contributed to the study that found SAMHD1 expression level can be used as a prognostic marker for ara-C treatment and that inhibition of SAMHD1 increases the sensitivity of AML cells to ara-C treatment. Finally, we identified a novel regulatory role for *NAP1L3* as a histone chaperone in self-renewal and differentiation of HSCs.

LIST OF SCIENTIFIC PAPERS

I. **The chromatin-remodeling factor CHD4 is required for maintenance of childhood acute myeloid leukemia.**

Yaser Heshmati, Gözde Türköz, Aditya Harisankar, Shabnam Kharazi, Johan Boström, Esmat Kamali Dolatabadi, Aleksandra Krstic, David Chang, Robert Månsson, Mikael Altun, Hong Qian and Julian Walfridsson. *Haematologica,* 2018, volume 103 (7), 1169-1181.

II. **Identification of GTF2IRD1 as a novel transcription factor essential for acute myeloid leukemia.**

Yaser Heshmati, Gözde Türköz, Marios Dimitriou, Aditya Harisankar, Johan Boström, Mikael Altun, Hong Qian, Nadir Kadri, Julian Walfridsson. *Manuscript*.

III. **The histone methyltransferase EHMT1 plays both independent and cooperative regulatory role in the maintenance of Acute Myeloid Leukemia**

Yaser Heshmati*, Gözde Türköz*, Emma Wagner, Aditya Harisankar, Johan Boström, Mikael Altun, Hong Qian and Julian Walfridsson. *Manuscript*.

*Authors contributed equally to this study

IV. **Targeting SAMHD1 with the Vpx protein to improve cytarabine therapy for hematological malignancies.**

Nikolas Herold, Sean G Rudd, Linda Ljungblad, Kumar Sanjiv, Ida Hed Myrberg, Cynthia B J Paulin, **Yaser Heshmati**, Anna Hagenkort, Juliane Kutzner, Brent D G Page, José M Calderón-Montaño, Olga Loseva, Ann-Sofie Jemth, Lorenzo Bulli, Hanna Axelsson, Bianca Tesi, Nicholas C K Valerie, Andreas Höglund, Julia Bladh, Elisée Wiita, Mikael Sundin, Michael Uhlin, Georgios Rassidakis, Mats Heyman, Katja Pokrovskaja Tamm, Ulrika Warpman-Berglund, Julian Walfridsson, Sören Lehmann, Dan Grandér, Thomas Lundbäck, Per Kogner1, Jan-Inge Henter, Thomas Helleday & Torsten Schaller. *Nature Medicine, 2017, Volume 23, 252-263*.

V. **The histone chaperone NAP1L3 is required for hematopoietic stem cell maintenance and differentiation.**

Yaser Heshmati, Shabnam Kharazi, Gözde Türköz, David Chang, Esmat Kamali Dolatabadi, Johan Boström, Aleksandra Krstic, Theodora Boukoura, Emma Wagner, Nadir Kadri, Robert Månsson, Mikael Altun, Hong Qian, Julian Walfridsson. *Scientific reports, 2018, volume 8(1)11202*.

RELATED PUBLICATIONS NOT INCLUDED IN THE THESIS

Distinct roles of mesenchymal stem and progenitor cells during the development of Acute Myeloid Leukemia in mice.
Pingnan Xiao, Lakshmi Sandhow, **Yaser Heshmati**, Makoto Kondo, Thibault Bouderlique, Monika Dolinska, Anne-Sofie Johansson, Mikael Sigvardsson, Marja Ekblom, Julian Walfridsson, and Hong Qian. *Blood advances, 2018, Volume 2, 1480-1494*.

Xeno-immunosuppressive properties of human decidual stromal cells in mouse models of alloreactivity in vitro and in vivo.
Behnam Sadeghi, **Yaser Heshmati**, Bita Khoein, Helena Kaipe, Mehmet Uzunel, Julian Walfridsson, Olle Ringden. *Cytotherapy, 2015, Volume 17, 1732-1745.*

CONTENTS

LIST OF ABBREVIATIONS

5-hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ADP	Adenosine diphosphate
AGM	Aorta-Gonad-Mesonephros
AML	Acute Myeloid Leukemia
ASXL1	Additional Sex Combs Like 1
BAD	Bcl-XL/Bcl-2-Associated Death
bHLH	basic-Helix-Loop-Helix
BM	Bone Marrow
BMI1	B Lymphoma Mo-MLV Insertion region 1
BMP4	Bone Morphogenetic Protein 4
BrdUrd	BromodeoxyUridine
C/EBPα	CCAAT/Enhancer Binding Protein alpha
CAR	CXC chemokine ligand (CXCL)12-Abundant Reticular
CAR	Chimeric Antigen Receptor
CARTs	CAR-transduced T cells
CBFA2	Core-Binding Factor subunit α-2
CBL	Casitas B-Lineage Lymphoma
CCL3	C-C Motif Chemokine Ligand 3
CD	Cluster of Differentiation
CEBPA	CCAAT Enhancer Binding Protein A
CFU	Colony Forming Unit
CFU-E/BFU-E	CFU-Erythrocytes
CFU-G/GM	CFU-Granulocytes/Macrophages
CFU-GEM	CFU-Granulocyte, Erythrocyte, Monocyte/macrophage
CFU-M	CFU-Macrophages

CH	Clonal Hematopoiesis
CHD4	Chromodomain Helicase DNA Binding Protein 4
CLP	Common Lymphoid Progenitors
CMP	Common Myeloid Progenitor
CN-AML	Cytogenetically Normal AML
CR	Complete Remission
CRISPR	Clustered Regularly Interspaced Short Palindrome Repeats
CRM1	Chromosome Region Maintenance 1
CXCL12	C-X-C Motif Chemokine Ligand 12
DNMT	DNA Methyltransferase
DNMT3A	DNA Methyltransferase 3A
DOT1L	Disruptor of Telomeric silencing 1-Like
DSBs	Double Strand Breaks
dsRNA	double strand RNA
EHMT1/2	Euchromatic Histone Lysine Methyltransferase 1/2
ELN	European LeukemiaNet
EMP	Erythroid and Myeloid Progenitors
ERK1/2	Extracellular signal-Related Kinase 1/2
ETV6	ETS Variant 6
FAB	French-America-British
FACS	Fluorescent Activated Cell Sorter
FDCP	Factor-Dependent Continuous Paterson Laboratories
FcR	Fragment crystallizable Receptor
FDA	Food and Drug Administration
FISH	Fluorescent In Situ Hybridization
FLT3	Fms-Like Tyrosine kinase 3
GAB1	GRB2-Associated Binder 1

GADD45a	Growth Arrest and DNA Damage Inducible Alpha
GFI1	Growth Factor Independent 1
GFP	Green Fluorescent Protein
GMP	Granulocyte-Monocyte Progenitor
GNAT	Glycine N-Acyltransferase-Like Protein 1
GRB2	Growth Factor Receptor-Bound2
GSEA	Gene Set Enrichment Analysis
GTF2IRD1	GTF2I Repeat Domain Containing 1
GVHD	Graft Versus Host Disease
HAT	Histone AcetylTransferase
HDAC	Histone Deacetylase
HDR	Homology-Directed Repair
HES1	Hairy and Enhancer of Split 1
HLA	Human Leukocyte Antigen
HLA-DR	Human Leukocyte Antigen D-related
HOTAIRM1	HOX antisense intergenic RNA myeloid 1
HOX	Homeobox
HOXA9	Homeobox A9
HSCs	Hematopoietic Stem Cells
HSPCs	Hematopoietic Stem and Progenitor Cells
HTS	High-Throughput Screen
IDH	Isocitrate dehydrogenase
IDH	Isocitrate Dehydrogenase
ITD	Internal Tandem Duplication
JARID1	Jumonji AT-Rich Interactive Domain 1
KLF1	Kruppel-Like Factor 1
LICs	Leukemia Initiating Cells

LMO2	LIM Domain Only 2
LMPP	Lymphoid-primed Multipotent Progenitors
lncRNA	long non-coding RNA
LSCs	Leukemic Stem Cells
LSD1	Lysine-Specific Demethylase 1
MAPK	Mitogen-Activated Protein Kinase
MDS	Myelodysplastic Syndromes
MDS/MPN	Myelodysplastic/Myeloproliferative Neoplasm
MEIS1	Myeloid Ecotropic Viral Integration Site 1
MFC	Multiparameter Flow Cytometry
MGF	Mas cell Growth Factor
MHC	Major Histocompatibility Complex
miRISC	miRNA-induced silencing complex
miRNA	microRNA
MLL	Mixed Lineage Leukemia
MPAL	Myeloid or mixed Phenotype Acute Leukemia
MPP	Multipotent Progenitors
MRD	Minimal Residual Disease
MSCs	Mesenchymal Stem Cells
NAP1L3	Nucleosome Assembly Protein 1 Like 3
NGS	Next Generation Sequencing
NHEJ	Non-homologous End Joining
NK	Natural killer
NPM1	Nucleolar Phosphoprotein Member 1
NSG-SGM3	NOD Scid IL2Rgnull-3-SCF/GM/IL3
Ocn	Osteocalcin
Osx	Osterix

PAM	Protospacer Adjacent Motif
PBX3	Pre-B-Cell Leukemia Homeobox 3
PcG	Polycomb-Group
PCR	Polymerase Chain Reaction
PHF6	PHD Finger Protein 6
PI	Propidium Iodide
piRNA	piwi-interacting RNA
PRC1/2	Polycomb Repressive Complex 1/2
pre-GM	pre-Granulocyte-Macrophage progenitors
PTD	Partial Tandem Duplications
PTH	Parathyroid Hormone
PU.1-AS	PU.1 antisense transcript
RB1	Retinoblastoma 1
RBC	Red Blood Cells
RFP	Red Fluorescent Protein
RING1/2	Really Interesting New Gene 1/2
RISC	RNA induced silencing complex
RNAi	RNA interference
rRNA	ribosomal RNA
RT-qPCR	Real-Time quantitative PCR
s-AML	secondary AML
SAMHD1	SAM and HD Domain 1
SBDS	Shwachman-Bodian-Diamond Syndrome
Sc	Scramble
scaRNA	small cajal body-specific RNA
SCF	Stem Cell Factor
SF3B1	Splicing Factor 3b Subunit 1

sgRNA	single guide RNA
SHC	Src Homology 2 Domain Containing
shRNA	short hairpin RNA
SIPA1	Signal-Induced Proliferation-Associated gene 1
SIV	Simian Immunodeficiency Virus (SIV)
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
SRSF2	Serine and Arginine rich Splicing Factor 2
STAT5a	Signal Transducer and Activator of Transcription 5A
Suz12	Suppressor of Zeste 12
t-AML	therapy-related AML
TAL1	T-Cell Acute Lymphocytic Leukemia 1
TdT	Terminal deoxynucleotidyl Transferase
TET2	Tet methylcytosine dioxygenase 2
TFs	Transcripton Factors
TK	Thymidine Kinase
TKD	Tyrosine Kinase Domain
TP53	Tumor Protein P53
TPO	Thrombopoietin
tRNA	transfer RNA
U2AF1	U2 Small Nuclear RNA Auxiliary Factor 1
UCBs	Umbilical Cord Blood Cells
VCAM-1	Vascular Cell Adhesion Molecule 1
VLPs	Virus-Like Particles
WBCs	White Blood Cells
WHO	World Health Organization

1 INTRODUCTION

1.1 HEMATOPOIETIC SYSTEM

The hematopoietic system includes the bone marrow (BM), spleen, lymph nodes and thymus, which are involved in the production of various blood cell types within the body. Specialized blood cells perform different functions in the body including oxygen supply, contributing to wound healing and protection from pathogens.

Oxygen is a vital component of all human cells, essential for cell growth and energy production. Red blood cells (RBCs) are responsible for oxygen transfer from the lung to all cells in the human body. Oxygen transport from the lung to the blood and uptake by cells is performed by passive diffusion (Krogh 1919). The protein hemoglobin in RBCs facilitates the transferring of 98% of oxygen in blood via reversible binding, whilst the rest of oxygen is found in free form in plasma and inside erythrocytes (Popel 1989).

Blood cells play an important role during hemostasis to stop bleeding, induce inflammation and prevent infection. In the wound healing process, numerous types of blood cells including platelets, neutrophils, monocytes, lymphocytes and dendritic cells are involved. The wound repair process is divided into three stages: inflammation, new tissue formation and remodeling (Gurtner, Werner et al. 2008). In the first stage, blood cells (immune cells and platelets) activate the coagulation cascade (hemostasis), inflammation pathways and prime anti-infection machinery. Platelets aggregate at the wound site and induce plug formation, after which insoluble fibrin forms a mesh to strengthen and stabilize the blood clot (Gale 2011).

Blood cells also contribute to both types of immunity, the innate and adaptive responses. The major blood cells and their products in the innate immune system include monocytes, macrophages, neutrophils and natural killer (NK) cells. The central players in the innate immune system are neutrophils, which are recruited and activated at the site of infection to eliminate pathogens (Witko-Sarsat, Rieu et al. 2000). NK cells are morphologically similar to B and T lymphocytes, but unlike lymphocytes they do not have specific antigen receptors. NK cells recognize abnormal cells through either FcR (immunoglobin receptors) or their receptors for MHC (major histocompatibility complex) class I (Parkin and Cohen 2001). The adaptive immune system consists of two major types of lymphocytes: T cell are involved in cell-mediated response and B cells are mainly responsible for antibodies-related (humoral) immunity.

1.1.1 Hematopoiesis

Hematopoiesis describes the procedure of formation, development and differentiation of blood cells. In a healthy adult human, the blood system continuously produces approximately 10^{11}-10^{12} blood cells daily (Beerman, Maloney et al. 2010, Doulatov, Notta et al. 2012, Harrison 1979). Here, the term of blood cells includes RBCs, platelets and white blood cells WBCs). The main hematopoietic cell types and their functions are summarized in Table 1.

The most common cells found in blood are RBCs, also named erythrocytes. In the human body, 2-3×10^6 RBCs are produced every second in the BM with a life span of 120 days. Macrophages in the spleen or liver remove old or damaged RBCs from the blood circulation.

Platelets originating from megakaryocytes play a role in hemostasis, thrombosis, inflammation and the immune system with an average life time of 8-10 days (Ho-Tin-Noe 2018).

WBCs are divided into two major subgroups: lymphoid and myeloid cells. The lymphoid cells consist of T cells, B cells and NK cells, which have roles in innate and adaptive immunity. The myeloid group includes monocytes, granulocytes (neutrophils, eosinophils and basophils), megakaryocytes and erythrocytes.

In mammalian adults, all blood cell types originate from a rare cell type in the BM termed hematopoietic stem cells (HSCs).

Table1. Hematopoietic cell types and their function.

Progenitors	Subtype	Function
Myeloid cells	Eosinophils	Involved in hypersensitivity and helminth infection
	Granulocytes (neutrophils)	Ingestion and destruction of microorganism
	Basophil	Inflammatory reactions and acute and chronic allergic development
	Macrophages	Derived from monocytes, respond to foreign material and release substances to stimulate other immune cells
	Megakaryocytes	Give rise to platelets
	Monocytes	Circulate in blood and migrate to tissue, they differentiate into macrophages
	Erythrocytes	Oxygen delivering
	Mast cells	Involved in allergy, anaphylaxis, wound healing, angiogenesis
Lymphoid cells	Cytotoxic T cells	Eradicate virus-infected cells as well as tumor cells
	T helper (T_H) cells	Produce cytokines to activate B cells and T cells
	Memory T cells	Previously encountered and respond to their cognate antigen and ready to respond faster and stronger to the same antigen
	B cells	Produce antibodies
	Dendritic cells	Process antigen and present to T cells
	Natural killer cells	Part of innate immune system to kill viral infected cells and cancer cells

1.1.2 Hematopoietic stem cells and differentiation

HSCs are characterized by a self-renewal capacity to generate more stem cells (clonal expansion) and their ability to differentiate into various kinds of progenitor cells (clonal extinction) (Pina and Enver 2007). Indeed, HSCs represent the top cellular hierarchy of blood cells, from which progenitors derive, that give rise to common precursor cells (Figure 1). Studies have shown that only approximately 1000 HSCs contribute to hematopoiesis and peripheral blood production (Catlin, Busque et al. 2011). Early *in vivo* HSC tracing experiments performed in mice, revealed that the average cell division time of a quiescent HSC is 57 days (Cheshier, Morrison et al. 1999). Interestingly, other studies showed that dormant HSCs divided every 145 days or five times in a mouse's lifespan (Wilson, Laurenti et al. 2008). Dormant HSCs (d-HSCs) are thought to only serve in injury situations.

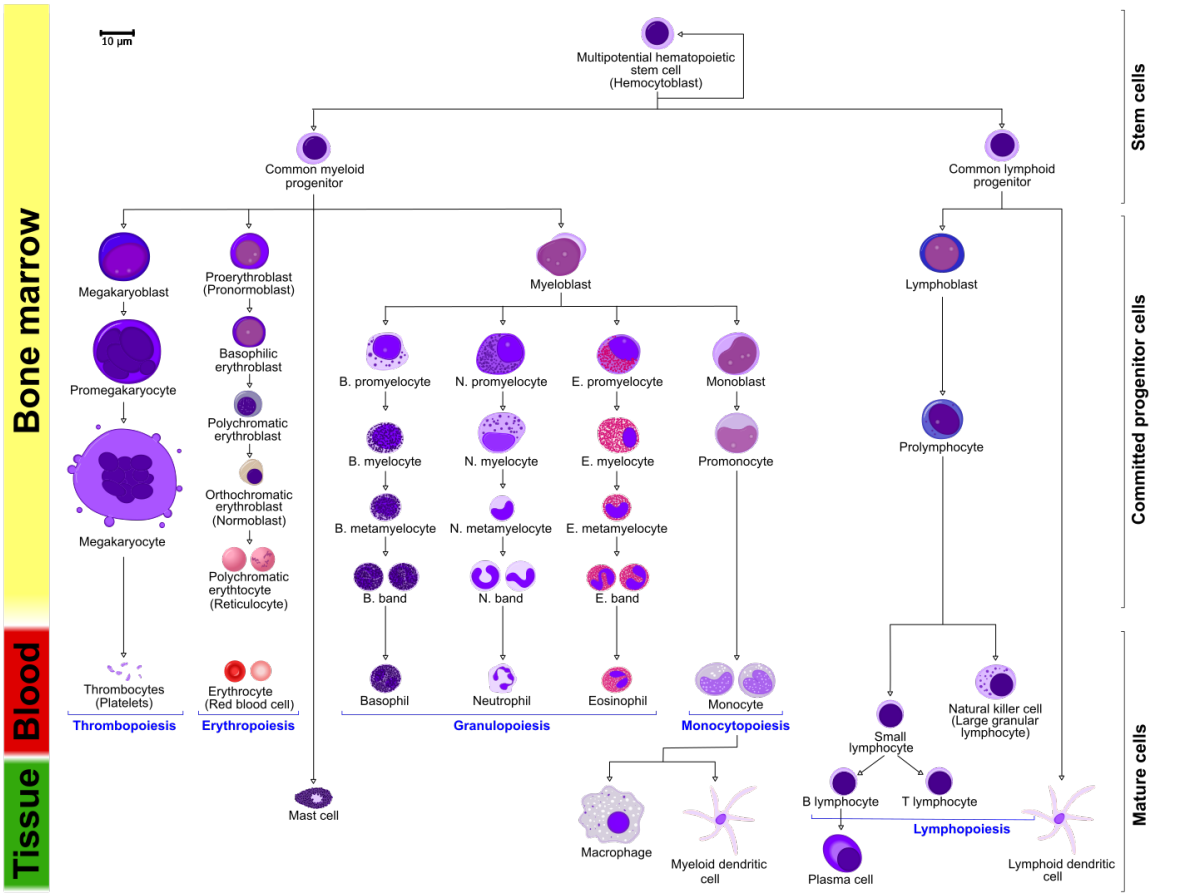

Figure 1. Hierarchy model of hematopoiesis. Hematopoietic stem cells (HSCs) with self-renewal capacity differentiate into progenitor cells and all mature blood cells. Figure reprinted with permission from the publisher (Wikipedia 2018).

In humans, it is not feasible to estimate the division time of HSCs by BrdUrd or H2-B labelling, but by using telomere length or the changing ratio of maternal/parental X-chromosome with age, the HSC replication rate was estimated to be on average once every 40-45 weeks (Catlin, Busque et al. 2011, Shepherd, Guttorp et al. 2004). However, considering mouse and human lifespans, the division number of HSCs is roughly similar between mice and humans.

Origin of HSCs

During embryogenesis, blood cells originate from the mesodermal layer. The first wave of blood cell development occurs in the yolk sac (day E7.5 in mouse) and is termed "primitive" or embryogenic hematopoiesis, as it produces primitive nucleated erythroid, macrophage and megakaryocytes progenitors (Figure 2) (Palis, Robertson et al. 1999, Tober, Koniski et al. 2007). The second hematopoietic wave in the yolk sac, embryo proper and allantois generates erythroid and myeloid progenitors (EMP); B-1a and T lymphocytes (Dzierzak and Bigas 2018). Thus, the formation of blood cells in the first and second wave occurs without existing of HSCs. In the mouse embryo, HSCs originate at day E9.5 from endothelial cells expressing vascular endothelial cadherin (Zovein, Hofmann et al. 2008).

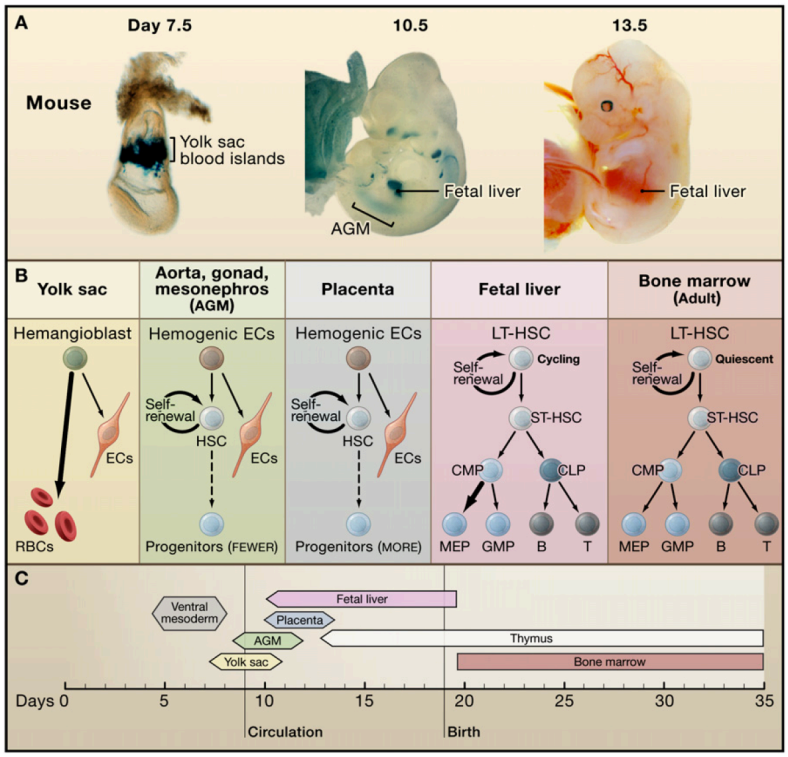

Figure 2. Development of hematopoiesis in mice.

A. The position of hematopoiesis first in yolk sac (YS) blood island then in aorta-gonad mesonephros (AGM) at day 10.5 and later in fetal liver, spleen and BM. **B**. The blood lineage hierarchy in each step of hematopoiesis. ECs (endothelial cells), RBCs (red blood cells), LTHSC (long-term hematopoietic stem cell), ST-HSC (short-termhematopoietic stemcell), CMP (common myeloid progenitor), CLP (common lymphoid progenitor), MEP (megakaryocyte-erythroid progenitor), GMP (granulocyte-monocyte progenitor). **C**. time frame and location of hematopoiesis. Figure reprinted with permission from the publisher (Orkin and Zon 2008).

Hematopoiesis converts to “definitive” in aorta-gonad mesonephros (AGM), fetal liver and eventually in the BM (adult hematopoiesis) (Dzierzak and Medvinsky 1995, Galloway and Zon 2003).

HSC Heterogeneity

For a long time, HSCs were thought to be one homogenous population which gave rise to multipotent progenitors (MPPs) which in turn differentiated into different lineages. However, recent findings demonstrate that HSCs are heterogonous. Indeed, single-cell transplantation of HSCs showed a broad range of variability in the reconstitution and self-renewal capacity of mouse HSCs (Dykstra, Kent et al. 2007, Morita, Ema et al. 2010, Yamamoto, Morita et al. 2013). Taking advantage of new *in vivo* imaging technology, HSCs are now classified as having long-, intermediate- and short-term self-renewal capacities based on their specific cell surface markers (Doulatov, Notta et al. 2012, Notta, Doulatov et al. 2011). Aside from their heterogeneity in self-renewal capacity, single-cell transplantation experiments also demonstrated that the majority of HSCs are biased towards certain lineages during differentiation and only a few can differentiate equivalently to produce all mature blood cells (Carrelha, Meng et al. 2018, Dykstra, Kent et al. 2007, Morita, Ema et al. 2010, Yamamoto, Morita et al. 2013). Additionally, single-cell RNA sequencing revealed the heterogeneity of RNA expression profiles amongst HSCs, which reflect their ultimate destination of differentiation (Adolfsson, Mansson et al. 2005, Velten, Haas et al. 2017). Thus, all current data suggest that transcriptional lineage programming determines HSC function and is associated with HSC lineage commitment.

Together, considering the limitations to isolate pure HSCs and using different definition and markers to identify HSCs in studies, HSCs are a heterogeneous population with varying RNA expression profiles, differing differentiation abilities and distinct reconstitution and self-renewal capacities.

Isolation of hematopoietic stem cells

As discussed above, HSCs are very rare, which makes their deep characterization difficult. For example, in human BM, where the HSCs reside, only 1 in a million cells represents a HSC (Wang, Doedens et al. 1997). So far, no unique marker for HSCs has been discovered.

For practical reasons, HSCs have mainly been studied in the mouse. To immune phenotypic isolation of mouse HSCs from the BM, HSCs were first defined as $Lin^{-}cKit^{+}Sca\text{-}1^{+}$ (LSK)

cells in 1992 (Ikuta and Weissman 1992). Among the LSK cells, functional studies revealed that the $CD34^-$ population was significantly enriched for long-term HSCs (Osawa, Hanada et al. 1996). Later, the HSCs could be further enriched to constitute 35-50% of the isolated cells, using an alternative sorting protocol defined as the SLAM phenotype ($CD150^+CD48^-$) (Kiel, Yilmaz et al. 2005).

To isolate and study HSCs in humans, The surface marker CD34 was suggested to enrich HSCs as it is found on less than 5% of all blood cells (Civin, Strauss et al. 1984). Although more than 99% of all human HSCs are $CD34^+$, several studies have shown the existence of $CD34^-$ HSCs (Ishii, Matsuoka et al. 2011). Further studies have shown that HSCs in humans can be further enriched in cells not expressing CD38 or CD45RA (Bhatia, Wang et al. 1997, Lansdorp, Sutherland et al. 1990). Another interesting marker is CD90 (Thy1) which has been used to obtain highly purified HSCs (Baum, Weissman et al. 1992). Therefore, $Lin^-CD34^+CD38^-CD90^+CD45RA^-$ is an immune phenotype that has been widely used to study and isolate human HSCs in the field, although this protocol is continuously being updated with additional markers. For example, including CD49f marker in $Lin^-CD34^+CD38^-CD90^+CD45RA^-$ cells led to further purification of long-term repopulating HSCs (Notta, Doulatov et al. 2011). Although the research field has not been able to isolate a completely pure population of HSCs, which might reflect their heterogeneity, we can now enrich them for further study of HSCs. A summary of specific markers used for isolation of each HSC and progenitor population in mouse and human is depicted in Figure 3.

Bone marrow niche

The concept of a niche for hematopoietic stem cells was first suggested in 1978 (Schofield 1978). This hypothesis proposed that HSCs associated with other cells in BM. BM is a semi-solid tissue located in the spongy or cancellous portions of the tibia, femur, ribs, sternum, vertebrae and pelvis. The BM niche regulates various HSC activities including self-renewal, differentiation, mobilization, and engraftment. The main cell types in the BM niche are osteolineage cells, perivascular cells, endothelial cells, adipocytes, macrophages and mesenchymal stem cells (MSCs) (Figure 4).

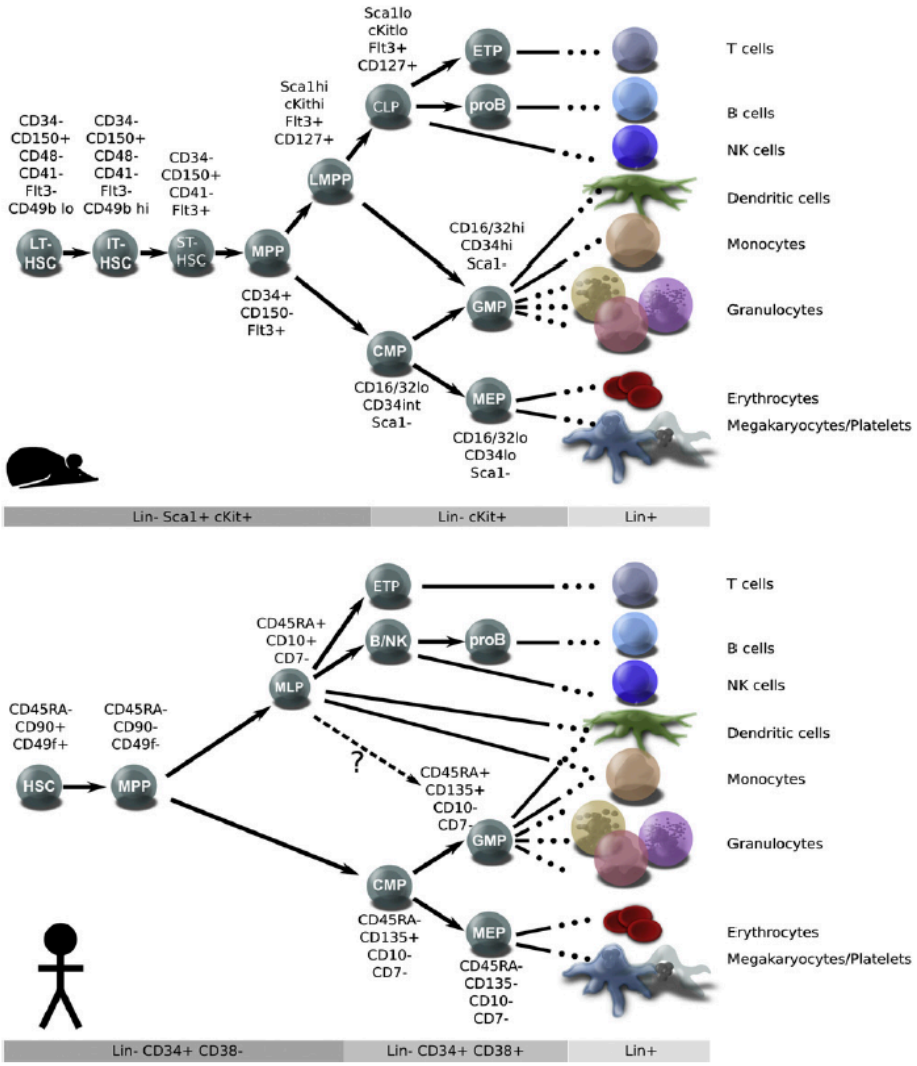

Figure 3. Cell surface markers of main classes of stem cells and progenitors in mouse and human. Figure reprinted with permission from the publisher (Doulatov *et al*. 2012).

It has previously been reported that various types of osteolineage cells reside in the BM with distinct roles regulating different lymphoid cells. Knockout of osteocalcin (Ocn)-expressing osteolineage cells results in a loss of T cells (Yu, Saez et al. 2015), whereas osterix-expressing (Osx^{+}) and Col(I)a2.3-expressing osteolineage cell deletion impairs the maturation of B cell progenitors (Visnjic, Kalajzic et al. 2004, Zhu, Garrett et al. 2007). Many HSCs localize adjacent to the blood vessels, which suggests that the perivascular region is crucial for HSC maintenance. In addition, MSCs also reside near vessels and can differentiate into osteolineage cells, chondrocytes, and adipocytes. Genetic deletion of MSCs in mice has recently been shown to affect the HSCs (Mendez-Ferrer, Battista et al. 2010), for example SIPA-1 deletion leads to the development of myelodysplastic/myeloproliferative neoplasm (MDS/MPN) phenotype in mice (Xiao, Dolinska et al. 2018). Another cell type which surrounds sinusoidal endothelial cells, or is located near to the endosteum, is CXC chemokine ligand (CXCL)12-abundant reticular (CAR) cells which co-localize with HSCs (Sugiyama,

Kohara et al. 2006). Ablation of CAR cells dramatically affects adipogenic and osteogenic differentiation, whilst decreased production of stem cell factor (SCF) and CXCL12 cytokines, subsequently caused a reduction in erythroid progenitors, cycling lymphoid cells and HSCs (Omatsu, Sugiyama et al. 2010).

Endothelial cells secrete specific growth factors in a paracrine-specific manner, known as angiocrine factors, and balance self-renewal and support expansion of hematopoietic stem and progenitor cells (HSPCs) over differentiation (Kobayashi, Butler et al. 2010). Moreover, they express adhesion molecules including P-selectin, E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (Mazo, Gutierrez-Ramos et al. 1998, Rafii, Mohle et al. 1997) which are important for HSCs homing into the BM. The percentage of adipocytes in the BM or fatty marrow increases with age in both humans and mice, yet HSC function decreases, implying that adipocytes negatively regulate HSCs (Yu and Scadden 2016).

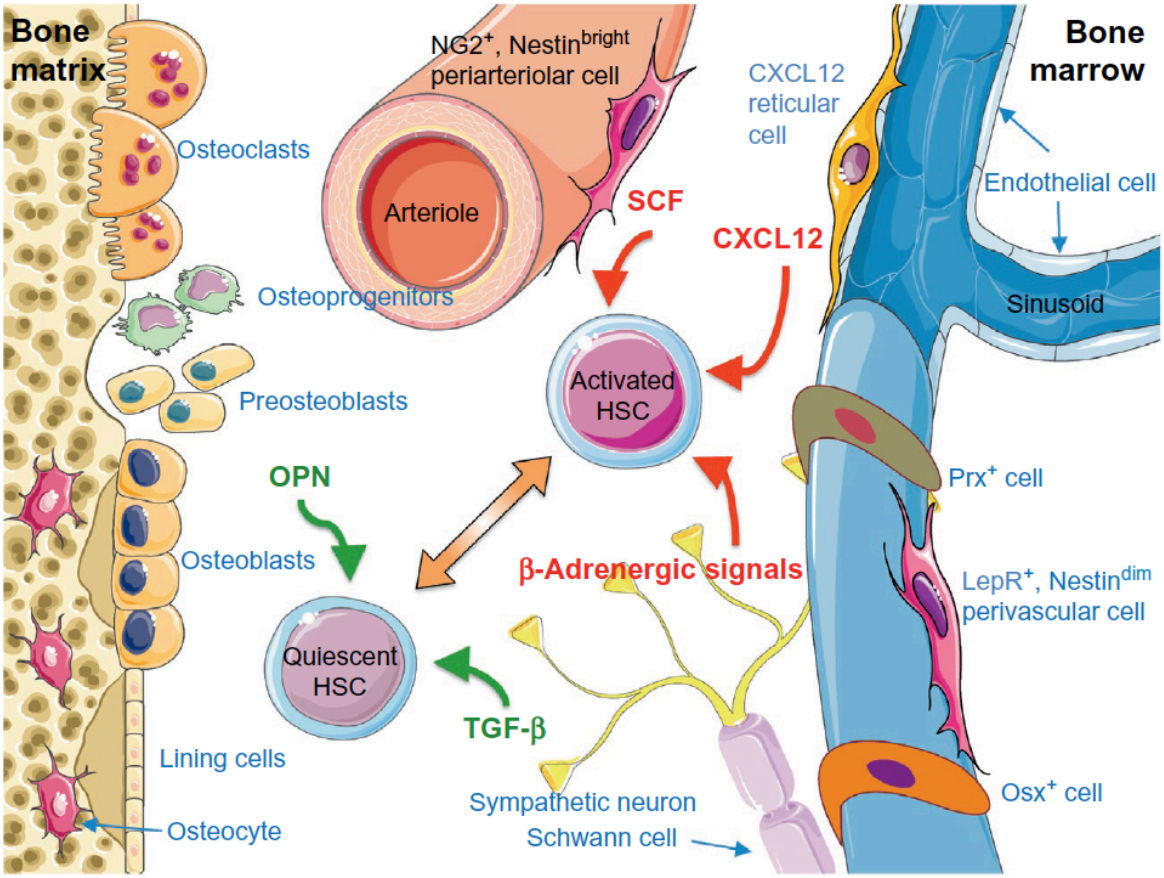

Figure 4. HSC niche in the adult bone marrow. Schematic of various stromal cells types and extrinsic signals including growth factors, cytokines, morphogens, extracellular matrix proteins, and adhesion molecules in the adult BM that contribute to the maintenance and regulation of HSCs. Figure reprinted with permission from the publisher (Yu and Scadden 2016).

Macrophages are another key cell type in the BM niche. Depletion of macrophages caused loss of osteoblasts, significant reduction of HSC-trophic cytokines and HSC mobilization into the blood (Winkler, Sims et al. 2010). Interestingly, macrophages supported erythroid lineage development (Palis 2016).

New findings have suggested that HSCs reside in different spots within the BM and depending on their location may have different functions. For example, some studies have shown that osteoblastic cells regulate HSCs via N-cadherin (Calvi, Adams et al. 2003, Zhang, Niu et al. 2003). Yoshihara suggested HSCs close to thrombopoietin-producing osteoblasts remain in a quiescent state (Yoshihara, Arai et al. 2007), whilst another study revealed that the vascular cells regulate HSCs migration to the vascular niche via CXCL12 (Kiel and Morrison 2006). Altogether, it seems that HSC localize to a specific area within the BM and are surrounded by hematopoietic as well as non-hematopoietic cells, which in turn provide a tonic regulation of HSCs. Therefore, any perturbation of these non-hematopoietic cells may cause abnormal function of HSCs.

1.1.3 Epigenetic regulation of hematopoiesis

The term “epigenetic” (Epi is Greek prefix means “over”, “outside of”) was suggested by Conrad Waddington in 1942 (Waddington 2012). Epigenetics refers to the stable and heritable modifications in gene activity that occur without any alteration in DNA sequence, including histone modification, DNA methylation and non-coding RNAs. Epigenetic regulation is a complex process by combination and interaction of many epigenetic modifiers which add “writers” or remove “erasers” modifications on histones or DNA and recognize and respond to the modifications “readers”.

In eukaryotes DNA is wrapped around a histone octamer called the nucleosome and nucleosome units fold into chromatin structure. Epigenetic regulatory factors modify chromatin structure in both a global and gene-specific manner by post-translational modification of histones and/or DNA methylation to provide or prevent access of transcription factors (TFs) to promoter regions. In other words, epigenetic modifications can result in either “loose” chromatin regions, called euchromatin, which are available for TFs or “tightly” packed chromatin regions, termed heterochromatin, which are inaccessible for transcription. For example, transcriptionally silenced regions in eukaryotic cells are associated with methylation of DNA in CpG regions and histone H3 dimethylated on lysine 9 (H3K9me2) and histone H3 trimethylated on lysine 27 (H3K27me3) whereas transcriptionally active regions contain high levels of H4 trimethylated on lysine 4

(H3K4me3) and lack DNA methylation (Li, Carey et al. 2007). Transcription of a gene occurs on naked DNA by TFs and RNA polymerase enzymes. Therefore, epigenetic mechanisms to alter chromatin and provide naked DNA for co-activators and TFs is the key step to determine cell identities.

The epigenetic landscape was first suggested by Waddington in 1957 to explain the role of epigenetic factors to canalize cells, originating from stem cells, during differentiation (Ferrell 2012). Therefore, epigenetic factors stabilize gene expression profile in cells and canalize cell-type identities. Transcription and epigenetic regulatory factors play crucial roles in keeping the balance between HSC maintenance and differentiation. However, differentiation in the hematopoietic system is not as simple as Waddington's original theory. First, in the beginning of hematopoiesis (the first and second wave) in the embryo stage, differentiated cells are made prior to and independently of HSCs. Furthermore, it was confirmed that by inducing expression of specific lineage TFs in hematopoietic cells, it is possible to switch the cells from one lineage to another (Graf 2002). Below, we will discuss the important epigenetic factors which play a role in hematopoiesis.

DNA methylation

DNA methylation was first suggested in 1975 as an epigenetic mechanism of imprinting to inactivate the X-chromosome in female cells (Holliday and Pugh 1975, Riggs 1975). In human cells, DNA methylation predominantly occurs in cytosine at the C5 position. DNA methylation mainly occurs in CpG dinucleotides (60-80% of all CpGs are methylated) at the promoter regions which influences transcriptional activity (Lister, Pelizzola et al. 2009, Saxonov, Berg et al. 2006).

Stem cell differentiation is linked to gradual methylation of CpG islands by DNA methyltransferases (Trowbridge and Orkin 2011). In hematopoiesis, different methylation patterns are significantly correlated with the global gene expression pattern and results in cell fate decisions (Ji, Ehrlich et al. 2010). DNA methylation might play a role as an epigenetic gatekeeper to retain a specific-cell lineage pattern during differentiation.

Two methyltransferases in particular, DNMT3a and DNMT3b are required for *de novo* methylation. During differentiation of HSCs, DNMT3a and DNMT3b are involved in the silencing of genes which regulate HSC self-renewal activity (Trowbridge and Orkin 2011). Hence, deletion of *DNMT3a* and *DNMT3b* in HSCs caused upregulation of self-renewal genes and subsequently increased HSC self-renewal and impaired HSC differentiation

(Challen, Sun et al. 2014). Another methyltransferase, DNMT1 preserves the DNA methylation patterns during DNA replication. DNMT1 also has an important role during HSC differentiation towards multipotent progenitors of a myeloid-restricted lineage (Trowbridge, Snow et al. 2009). Genes that are important in maintaining HSCs, including *Meis1*, *Hoxa9* and *Prdm16*, are heavily methylated and so transcriptionally silenced in progenitor cells during differentiation (Kosan and Godmann 2016). Whereas, active genes in differentiated cells are found to be methylated in HSCs and selectively become demethylated during the lineage commitment process. For example, *Gadd45a* a critical gene in myeloid development was found to be upregulated and demethylated in cells transitioning from common myeloid progenitor (CMP) to granulocyte-monocyte progenitor (GMP) stage whereas it is methylated and silenced in HSCs (Ji, Ehrlich et al. 2010).

Histone modifications

There are many post-translational histone modifications which influence chromatin structure including acetylation, methylation, phosphorylation, ubiquitinylation, sumoylation, adenosine diphosphate (ADP) ribosylation, and deamination (Kouzarides 2007). These modifications are associated with various processes including transcription regulation, DNA repair, DNA replication and condensation (Kouzarides 2007). Histone-modifying enzymes are summarized in Table 2.

There are some important histone-modifying enzymes in hematopoiesis which play key roles in HSC function including self-renewal and differentiation. Important histone acetyltransferase (HAT) that transfer acetyl groups to specific lysine residue of histones in hematopoiesis are p300/CBP (CBP and p300), MYST (Tip60, MOZ, MORF, HBO1 and HMOF), and GNAT (PCAF, Gnc5 and ELP3) (Sun, Man et al. 2015). P300 is associated with promoting differentiation of HSCs by acetylation of the *C-Myb* promoter region, whereas CBP acetylates *Gfi1b* and promotes self-renewing of HSCs and blocks differentiation (Sun, Man et al. 2015).

Polycomb-group (PcG) proteins are histone modifiers which exist in two main complexes; polycomb repressive complex 1 (PRC1) and 2 (PRC2). They have been shown to be involved in gene repression in HSCs during self-renewal and differentiation (Radulovic, de Haan et al. 2013).

Table 2. Histone-modifying enzymes. Table adapted with permission from the publisher (Kouzarides 2007).

Function	Name	Residues modified
Acetyltransferase	HAT1	H4 (K5, K12)
	CBP/P300	H3 (K14, K18), H4 (K5, K8), H2A (K5), H2B (K12, K15)
	PCAF/GCN5	H3 (K9, K14, K18)
	TIP60	H4 (K5, K8, K12, K16), H3K14
	HB01	H4 (K5, K8, K12)
Deacetylase	SirT2	H4K16
Lysine Methyltransferase	SUV39H (1-2)	H3K9
	GLP/EHMT1	H3K9
	G9a/EHMT2	H3K9
	ESET	H3K9
	CLL8	H3K9
	MLL (1-5)	H3K4
	SET1 (A-B)	H3K4
	ASH1	H3K4
	SET2	H3K36
	NSD1	H3K36
	SYMD2	H3K36
	DOT1	H3K79
	Pr-SET 7/8	H4K20
	SUV4 20H(1-2)	H4K20
	EZH2	H3K27
	RIZ1	H3K9
Lysine Demethylase	LSD1/BHC110	H3K4
	JHDM1 (a-b)	H3K36
	JHDM2 (a-b)	H3K9
	JMJD2A/JHDM3A	H3 (K9, K36)
	JMJD2B	H3K9
	JMJD2C/GASC1	H3 (K9, K36)
	JMJD2D	H3K9
Arginine Methyltransferase	CARM1	H3 (R2, R17, R26)
	PRMT4	H4R3
	PRMT5	H3R8, H4R3
Serine/Thrionine Kinase	Haspin	H3T3
	MSK (1-2)	H3S28
	CKII	H4S1
	Mst1	H2BS14
Ubiquitilases	Bmi/Ring1A	H2AK119
	RNF20/RNF40	H2BK120
Proline Isomerase	ScFPR4	H3 (P30, P38)

PRC2 includes the enhancer of zeste (E(z)), Suppressor of zeste 12 (Suz12) and Extra sex combs (Esc). Overall, PRC2 is responsible for the methylation of H3K27 (H3K27me2 and H3K27me3) at the promoter sites which is associated with transcriptional repression of target genes. PRC1 consists of BMI1, RING1/2, MEL-18, RAE28/MPH1, and M33/CBX2. The PRC1 complex binds to H3K27me3, which is established by PRC2, and stabilizes gene silencing by H2A ubiquitination (H2AK119ub1). Ubiquitination of H2A as the last step of

gene repression is thought to inhibit RNA pol II in the initiation or elongation phase of transcription (Radulovic, de Haan et al. 2013). Deletion of *Bmi1*, part of PRC1, in mice caused a severe reduction in the number of long-term HSCs (Park, Qian et al. 2003), whilst deletion of *Mel-18* induced self-renewing of HSCs and a defect in B cells, suggesting a role for PRC1 in self-renewal and differentiation of HSCs (Akasaka, Tsuji et al. 1997, Kajiume, Ninomiya et al. 2004).

Mixed Lineage Leukemia (MLL), lysine methyl transferase enzyme, plays an important role in the second wave of embryo hematopoiesis, AGM and further HSC development (Orkin 2000). MLL is associated with transcriptional activation by trimethylation of H3 (H3K4me3) at downstream target genes, particularly *HOX* genes.

Two main members of the histone demethylase family are Lysine-specific demethylase 1 (LSD1) and the Jumonji C (JmjC) domain-containing family. LSD1 is part of a complex with histone deacetylases (HDACs) and CoREST and acts as a transcriptional repressor to downregulate expression of target genes by removing one or two methyl groups from H3K4 or H3K9. Several JmjC domain proteins have been found including JHDM1, JHDM2, JMJD2 and Jumonji AT-rich interactive domain 1 (JARID1). Jarid1b is highly expressed in HSCs and is important for HSC self-renewal (Stewart, Albert et al. 2015).

Some histone methylations such as H3K4, H3K36, and H3K79 are linked with gene activation, whereas methylation on H3K9, H3K27, and H4K20 results in transcription inhibition. Interestingly, many promoters in stem cells co-exist alongside transcriptional activation (H3K4me3) and repression marks (H3K27me3). These bivalent domains allow stem cells to rapidly activate or silence genes during differentiation (Voigt, Tee et al. 2013).

Non-coding RNA

RNAs that do not translate to proteins are defined as non-coding RNAs, which include ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small cajal body-specific RNA (scaRNA), piwi-interacting RNA (piRNA), microRNA (miRNA) and long non-coding RNA (lncRNA). Some non-coding RNAs, in particular miRNA and lncRNA, play a role in gene regulation and are therefore regarded as part of the epigenetic regulators.

miRNAs are short RNAs containing 21-24 bases and are responsible for post-transcriptional gene silencing by pairing to the complement region of target mRNA and recruiting miRNA-induced silencing complexes (miRISC) (Fabian and Sonenberg 2012). Several reports have

shown that miRNAs play a pivotal function in hematopoiesis. In 2004, miRNA-181 was found to play a role as a regulatory factor in B cell differentiation (Chen, Li et al. 2004). In addition, growth factor independent-1 (Gfi1); a critical transcription factor for granulocytic differentiation is regulated by miR-21 (Velu, Baktula et al. 2009). In erythroid cell differentiation miR-92a and miR-17 targets regulatory erythroid genes (Li, Vecchiarelli-Federico et al. 2012). miRNAs are not only important during differentiation but are also crucial in the regulation of HSPC expansion, for example overexpression of miR-17-92 induced proliferation of multipotent hematopoietic progenitors (Li, Vecchiarelli-Federico et al. 2012).

lncRNAs contain more than 200 bases and it is estimated that 10,000-60,000 lncRNAs are expressed in human cells (Wilkes, Repellin et al. 2017). lncRNAs regulate gene expression at various steps including mRNA processing, during translation or protein synthesis, modification of mRNA stability, miRNA inhibition and as a scaffold to facilitate mRNA synthesis (Geisler and Coller 2013). Numerous studies have linked lncRNAs to hematopoiesis. For example, over 100 lncRNAs are exclusively expressed during erythroid development, some of which target the transcription factors *GATA*, T cell acute lymphocytic leukemia protein 1 (*TAL1*) or Kruppel-like factor 1 (*KLF1*) (Geisler and Coller 2013). During myeloid development, PU.1 antisense transcript (PU.1-AS) was previously identified to target *PU.1* mRNA and downregulates *PU.1* transcription factor (Ebralidze, Guibal et al. 2008). Similarly, HOX antisense intergenic RNA myeloid 1 (HOTAIRM1) was identified as a myeloid-specific lncRNA which is highly expressed during granulocytic differentiation, whilst HOTAIRM1 suppresses *HOXA1* and *HOXA4* during myeloid differentiation (Zhang, Lian et al. 2009). Interestingly, more than 3000 lncRNAs have been found to be expressed during lymphoid development, suggesting that it is possible to characterize different lymphoid committed cells by the lncRNA expression patterns (Casero, Sandoval et al. 2015).

Altogether, these studies indicate the important role of non-coding RNAs as part of the epigenetic regulatory compartment in HSC function and during differentiation of progenitor cells.

1.1.4 Transcription factors involved in hematopoiesis

Another intrinsic element which determines the identity of cells is the particular TF network to induce expression of lineage-specific genes. Critical TFs in hematopoiesis, contain nearly all groups of DNA-binding proteins rather than favoring a specific class. Some TFs are required for the development and maintenance of HSCs, some for the differentiation process

and some are involved in both. For example *MLL*, *Runx1*, *TEL/ETV6*, *SCL/tal1* and *LMO2* are "HSCs TFs" but *PU.1*, *Gfi-1*, *C/EBPα* are more lineage-restricted factors (Orkin and Zon 2008). A summary of some important TFs in hematopoiesis is depicted in Figure 5.

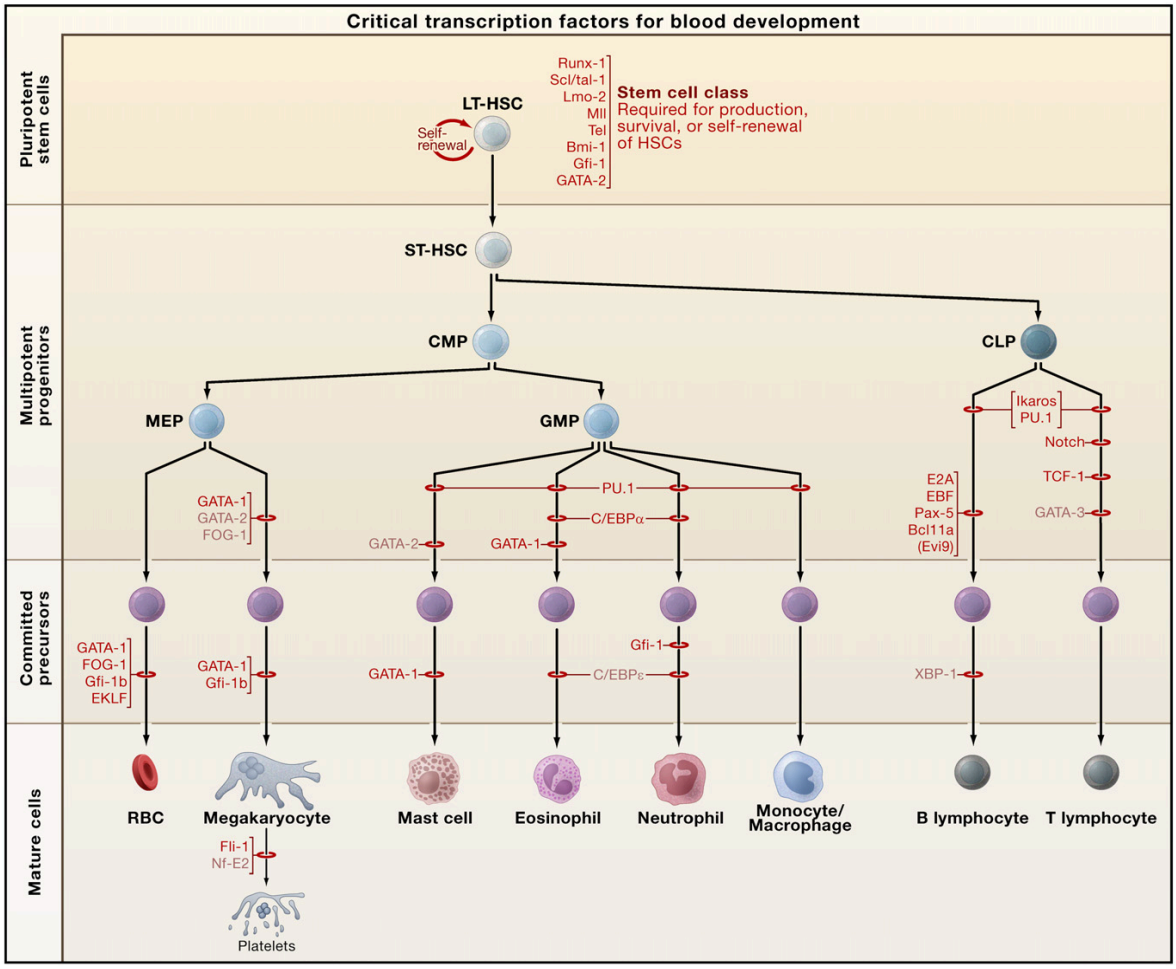

Figure 5. Important transcription factors in hematopoiesis. Important TFs in each stage depicted in red. The TFs in light font have not yet been identified translocated or mutated in human/mouse hematologic malignancies but the rest are associated with hematopoietic malignancies. LT-HSC (long-term hematopoietic stem cell), ST-HSC (short term hematopoietic stem cell), CMP (common myeloid progenitor), CLP (common lymphoid progenitor), MEP (megakaryocyte/erythroid progenitor), GMP (granulocyte/macrophage progenitor) RBCs (red blood cells). Figure reprinted with permission from the publisher (Orkin and Zon 2008).

Factors required for development and maintenance of HSCs

Development and maintenance of HSCs is pre-determined by a series of TFs, which are influenced by the microenvironment, signaling pathways and epigenetic regulators. During the emergence of HSCs, signaling molecules originating from the adjacent germ cell layer induce expression of crucial TFs required for this step. Therefore, physical interaction between primitive endoderm and adjacent mesoderm and subsequently endodermal signaling are required for the development of hematopoietic cells during the embryo stage (Belaoussoff, Farrington et al. 1998). Some important TFs required for programming the ventral mesoderm toward HSCs are basic-helix-loophelix (bHLH), SCL/TAL-1 and LMO2. The absence of these factors leads to defects in both endothelial and hematopoietic cells (Kim and Bresnick 2007). During the yolk stage, they are also required for development of blood cells, therefore they are pivotal for the development of primitive and definitive HSCs.

One of the key TFs for definitive hematopoiesis is Runx1, which is expressed during the first wave of hematopoiesis in mesoderm (North, Gu et al. 1999). Zebrafish models have shown that *Runx1*, *Lmo2* and *Scl* are regulated by Notch signaling, which induce expansion of HSCs (Burns, Traver et al. 2005). In adult human blood cells, Runx1 is expressed in all blood cells except erythrocytes (North, Stacy et al. 2004). *Runx1* and *Runx3* double-knockout caused BM failure and myeloproliferative disorder in mice via the non-transcriptional function of *Runx* in DNA repair (Wang, Krishnan et al. 2014). Apart from Runx1, another important factor for fetal HSCs is Sox17, which is uniquely expressed in HSCs during embryogenesis. Deletion of *Sox17* results in a lack of adult HSCs (Kim, Saunders et al. 2007).

Other TFs involved in hematopoiesis are *HOX* genes which classify in four clusters: *HOXA*, *HOXB*, *HOXC* and *HOXD*. HOX TFs are homeodomain-containing TFs which were originally characterized in Drosophila, yet with important roles in hematopoiesis (Shah and Sukumar 2010). They are expressed in HSCs and progenitors with specific expression profile patterns, dependent upon the cell lineage. For instance *HOXB3*, *HOXB4* and *HOXA9* are significantly expressed in uncommitted hematopoietic cells, while *HOXB8* and *HOXA10* are activated in myeloid-committed cells (Alharbi, Pettengell et al. 2013). Overexpression of *HOX* genes most often causes HSPC expansion and blockade of differentiation. Previously, *Hoxb6* overexpression has been demonstrated to increase the number of HSCs and myeloid progenitors whilst blocking erythropoiesis and lymphopoiesis (Fischbach, Rozenfeld et al. 2005).

The functional mechanism of *HOX* genes to regulate hematopoiesis is not yet clearly defined. However, some studies have suggested downstream targets of HOX TFs which are crucial in

hematopoiesis. For example, *HOXA9* activates expression of other *HOX* genes (*HOXA7* and *HOXA10*), *PBX3*, *MEIS1*, *Kit*, *Flt3* and *Sox4* which are important in hematopoiesis. Other important downstream target genes which are regulated by *HOX* genes are *GATA1*, *C-MYC* and *RUNX2* (Alharbi, Pettengell et al. 2013).

As mentioned above, one important upstream regulator of *HOX* is MLL, which directly regulates transcription of HOX genes. Another group of factors which directly regulate *HOX* genes are *CXD* genes (*CDX1*, *CDX2* and *CDX4*) (Brooke-Bisschop, Savory et al. 2017). Deletion of *Cdx1* and *Cdx2* in mice was shown to impair primitive hematopoiesis and resulted in a lack of primitive erythrocytes as well as an abnormal yolk sac vasculature (Brooke-Bisschop, Savory et al. 2017). Therefore, the *CDX* genes are also required for normal HSC function.

Important TFs for HSCs differentiation

Two crucial factors which are not only important for HSC generation, but also important for differentiation of HSCs are GATA-2 and Runx-1. Inducible deletion of *Runx-1* in BM cells showed that *Runx-1* is essential for development of megakaryocytes and T and B cell differentiation, but is not essential for normal myeloid development (Ichikawa, Asai et al. 2004). Other studies, however, show that *Gata2* contributes to the generation and long-term maintenance of HSCs (de Pater, Kaimakis et al. 2013). Several pathways upstream of *Gata2* and *Runx1* regulate their expression at different stages of the HSC development and differentiation. The important regulatory pathways for GATA2 and RUNX1 are NOTCH (Guiu, Shimizu et al. 2013), BMP4 (Walmsley, Ciau-Uitz et al. 2002) and ETS/EGR (Taoudi, Bee et al. 2011). There are also some myeloid factors which are expressed in GMPs such as C/EBPα (Orkin and Zon 2008).

Another member of the GATA family which plays a crucial role in hematopoiesis is GATA1. Expression of *GATA1* is important for erythropoiesis (Fujiwara, Browne et al. 1996). Specific deletion of *Gata1* in megakaryocytes reduced platelet numbers and impaired the differentiation of megakaryocytes, which in turn increased numbers of immature megakaryocytes in the BM and spleen (Kuhl, Atzberger et al. 2005, Shivdasani, Fujiwara et al. 1997). Additionally, *GATA1* has also been shown to have an important function in the development of mast cells, eosinophils, basophils, and dendritic cells (Crispino and Horwitz 2017).

Activation of lineage-specific TFs determine differentiation of HSCs and usually the activated TFs in turn act as suppressors for other lineage-specific TFs (Orkin and Zon 2008). For instance, GATA-1 and PU.1 physically interact and antagonize the function of each other (Rhodes, Hagen et al. 2005). Expression of FOG in multipotent cells suppresses the eosinophil factor C/EBPbeta and yet during the eosinophil lineage commitment, C/EBPbeta blocks expression of FOG (Querfurth, Schuster et al. 2000). The TFs FLI-1 and EKLF cross-antagonise their respective functions during megakaryocyte or erythroid development (Starck, Cohet et al. 2003). Moreover, GFI-1 physically interacts with and suppresses PU.1, inducing macrophage differentiation (Dahl, Iyer et al. 2007). Interestingly, in HSC differentiation, during the phenomenon called "the GATA switch", GATA2 activates GATA1 but after activation, GATA1 negatively regulates the expression of GATA2 (Grass, Boyer et al. 2003).

Altogether, the data support the fundamental role of TFs in hematopoiesis and Interestingly, somatic mutation or chromosomal translocations of the majority of TFs within the hematopoietic system are associated with hematopoietic malignancies.

1.2 ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is a heterogeneous hematological malignancy which is characterized by clonal expansion of abnormal or immature myeloid progenitors in the BM and peripheral blood (O'Donnell, Abboud et al. 2012).

AML is the most common subtype of acute leukemia in adults, covering around 80% of all leukemia cases in adults (Yamamoto and Goodman 2008). The AML incidence rate is 1.3 per 100,000 in the population younger than 65 years old and increases with age to 12.2 patients per 100,000 in the population over 65 years old in the USA (De Kouchkovsky and Abdul-Hay 2016). Despite the development of therapeutic drugs and improvement of supportive care over the past 50 years, AML is only curative in 35-40% of adult patients under 60 years of age and 5-15% in patients over 60 years old (Dohner, Weisdorf et al. 2015). Therefore, there is a great demand for more efficient therapeutic strategies in AML.

1.2.1 Diagnosis of AML

The general symptoms of AML patients result from an accumulation of poorly differentiated myeloid cells in the BM and peripheral blood leading to BM failure, anemia, thrombocytopenia, fatigue, anorexia, fever, night sweats and weight loss. Many symptoms

of AML are as a consequence of normal blood deficiency due to leukemia cells outcompeting normal cells in the BM. Therefore, patients do not have enough RBCs, WBCs and platelets.

Preliminary diagnosis of AML is based on morphology, notably the presence of 20% or more blast cells in at least 200 counted leukocytes on blood smear and 500 on speculated BM smear, with the exception of AML with t(15;17), t(8;21), inv(16), or t(16;16). (Dohner, Estey et al. 2017, Dohner, Estey et al. 2010). The nuclei of AML blasts are large in size and typically several nucleoli can be found in one AML cell.

Following morphology, subsequent diagnostic methods for AML are based on surface phenotyping. AML cells express CD markers that exist on normal myeloid cells, such as CD13, CD33 and CD34 (Campos, Guyotat et al. 1989). However, other CD markers might be expressed on AML cells dependent upon the AML subtype or stage of AML differentiation (Dohner, Estey et al. 2017). For example, AML cells may express CD4, CD14, CD11b, CD36 and CD64 (monocyte markers), CD36, CD71 and CD235a (erythroid markers), CD41 and CD61 (megakaryocyte markers). Moreover, AML cells may also express specific T or B cells markers; CD7, CD19, terminal deoxynucleotidyl transferase (TdT) and human leukocyte antigen-antigen D related (HLA-DR), (Dohner, Estey et al. 2017).

Finally, conventional cytogenetic analysis of AML cells is the next mandatory method for diagnosis and classification of AML cells which is based on chromosomal abnormalities. Fluorescence in situ hybridization (FISH) and new sequencing technologies are used to detect fusion genes (McKerrell, Moreno et al. 2016), following which molecular genetic screening for known mutations such as *NPM1*, *CEBPA*, *RUNX1*, *FTL3*, *TP53* and *ASXL1* should be done. The summary of tests or procedures for accurate diagnosis of AML is provided in Table 3 (Dohner, Estey et al. 2017).

Table 3. Summary of tests and procedures to diagnose AML.

Cellular assays
Blood count
Bone marrow aspirate
Immunophenotyping
Genetic analysis
Cytogenetics
Screening for mutations: NPM1, CEBPA, RUNX1, FLT3, TP53, ASXL1
Screening for fusion genes: PML-RAPA, CBFB-MYH11, RUNX1-RUNX1T1, BCR-ABL1, MLL-X
Additional procedures
Medical history including exposure to toxic agents, prior malignancy and therapy, smoking
Family history to check germ line predisposition of myeloid neoplasms

1.2.2 Classification of AML

Several different classification systems have been suggested over the years based on morphologic analysis of blood smears and BM samples, expression levels of cell-surface or cytoplasmic markers using flow cytometry, cytogenetic karyotype tests and screening of genes that are important for AML development and maintenance.

As of 1970, AML was classified into eight major subtypes (M0-M7) according to the French-American-British (FAB) system, which is based on the morphology and immune phenotype of AML cells (Bennett, Catovsky et al. 1976). More recently, AML has been classified according to the world health organization (WHO) classification in 2008, which is based on leukemia-associated chromosomal translocations and inversions (Sabattini, Bacci et al. 2010). According to the WHO classification, AML can be divided in seven subtypes: (1) AML with chromosomal abnormalities, (2) AML with myelodysplasia-related changes, (3) therapy-related myeloid neoplasms, (4) AML not otherwise specified (NOS) (similar to FAB Classification M0–M7 with others such as acute megakaryoblastic leukemia (AMKL), acute panmyelosis (APMF) with myelofibrosis, and pure erythroleukemia), (5) myeloid sarcoma, (6) myeloid proliferations related to Down syndrome, and (7) blastic plasmacytoid dendritic cell neoplasm (Vardiman, Thiele et al. 2009). The most recent WHO classification was updated in 2016 (Table 4) (Arber, Orazi et al. 2016). In the updated version, a new class "myeloid neoplasms with germ line predisposition" was added (Table 5); for example, germline *CEBPA* mutations frequently detected in AML patients who need special genetic counselling for their families (DiNardo, Bannon et al. 2016, Pabst, Eyholzer et al. 2008).

Presently, genetic analyses of AML samples by next generation sequencing (NGS) has provided further information and better views into AML classification (Grimwade, Ivey et al. 2016, Papaemmanuil, Gerstung et al. 2016).

AML can also be classified based on etiology into three categories: (1) de novo AML, (2) therapy-related AML (t-AML), and (3) secondary AML (s-AML) originated from MDS (myelodysplastic syndromes) or other myeloid proliferative disorder (Lindsley, Mar et al. 2015).

Table 4. WHO classification of AML and related precursor neoplasms and acute leukemia. Table adapted with permission from the publisher (Dohner, Estey et al. 2017).

AML with recurrent neoplasms
• AML with t(8;21)(q22;q22.1); RUNX1-RUNX1T1
• AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
• Acute promyelocytic leukemia with PML-RARA[1]
• AML with t(9;11)(p21.3;q23.3); MLLT3-KMT2A[2]
• AML with t(6;9)(p23;q34.1); DEK-NUP214
• AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1)
• AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); RBM15-MKL1[3]
• Provisional entity: AML with BCR-ABL1
• AML with mutated NPM1[4]
• AML with biallelic mutations of CEBPA[4]
• Provisional entity: AML with mutated RUNX1
AML with myelodysplasia-related changes[5]
Therapy-related myeloid neoplasms[6]
AML, NOS
• AML with minimal differentiation
• AML without maturation
• AML with maturation
• Acute myelomonocytic leukemia
• Acute monoblastic/monocytic leukemia
• Pure erythroid leukemia[7]
• Acute megakaryoblastic leukemia
• Acute basophilic leukemia
• Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
• Transient abnormal myelopoiesis
• Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm
Acute leukemias of ambiguous lineage
• Acute undifferentiated leukemia
• MPAL with t(9;22)(q34.1;q11.2); BCR-ABL1[8]
• MPAL with t(v;11q23.3); KMT2A rearranged
• MPAL, B/myeloid, NOS
• MPAL, T/myeloid, NOS

MPAL, mixed phenotype acute leukemia; NK, natural killer.

1. Other recurring translocations involving RARA should be reported accordingly: AML with t(11;17)(q23;q12); ZBTB16-RARA, t(11;17)(q13;q12); NUMA1-RARA, t(5;17)(q35;q12); NPM1-RARA, or AML with STAT5B-RARA.

2. Other translocations involving KMT2A (MLL) should be reported accordingly: AML with t(6;11)(q27;q23.3); MLLT4-KMT2A, AML with t(11;19)(q23.3;p13.3); KMT2A-MLLT1, t(11;19)(q23.3;p13.1); KMT2A-ELL, t(10;11)(p12;q23.3); MLLT10-KMT2A.

3. Rare leukemia most commonly occurring in infants.

4. Diagnosis is made irrespective of the presence or absence of multilineage dysplasia.

5. At least 20% blood or marrow blasts AND any of the following: previous history of MDS or MDS/MPN; myelodysplasia-related cytogenetic abnormality; multilineage dysplasia; AND absence of both prior cytotoxic therapy for unrelated disease and aforementioned recurring genetic abnormalities. Cytogenetic abnormalities sufficient to diagnose AML with myelodysplasia-related changes are: Complex karyotype (defined as 3 or more chromosomal abnormalities in the absence of 1 of the WHO-designated recurring translocations or inversions, that is, t(8;21), inv(16) or t(16;16), t(9;11), t(v;11)(v;q23.3), t(6;9), inv(3) or t(3;3); AML with BCRABL1); Unbalanced abnormalities: 27 or del(7q); 25 or del(5q); i(17q) or t(17p); 213 or del(13q); del(11q); del(12p) or t(12p); idic(X)(q13); Balanced abnormalities: t(11;16)(q23.3;p13.3); t(3;21)(q26.2;q22.1); t(1;3)(p36.3;q21.2); t(2;11)(p21;q23.3); t(5;12)(q32;p13.2); t(5;7)(q32;q11.2); t(5;17)(q32;p13.2); t(5;10)(q32;q21.2); t(3;5)(q25.3;q35.1).

6. Cases should be classified with the related genetic abnormality given in the diagnosis.

7. The former subgroup of acute erythroid leukemia, erythroid/myeloid type (≥50% bone marrow erythroid precursors and ≥20% myeloblasts among nonerythroid cells) was removed; myeloblasts are now always counted as percentage of total marrow cells. The remaining subcategory AML, NOS, pure erythroid leukemia requires the presence of >80% immature erythroid precursors with ≥30% proerythroblasts.
8. BCR-ABL1 leukemia may present as MPAL; treatment should include a tyrosine kinase inhibitor.
Table reprinted with permission from the publisher (Döhner *et al*. 2017).

Table 5. WHO classification of AML with germ line predisposition. Table adapted with permission from the publisher (Dohner, Estey et al. 2017).

Myeloid neoplasms with germ line predisposition without a preexisting disorder or organ dysfunction
• AML with germ line CEBPA mutation • Myeloid neoplasms with germ line DDX41 mutation[1]
Myeloid neoplasms with germ line predisposition and preexisting platelet disorders
• Myeloid neoplasms with germ line RUNX1 mutation[1] • Myeloid neoplasms with germ line ANKRD26 mutation[1] • Myeloid neoplasms with germ line ETV6 mutation[1]
Myeloid neoplasms with germ line predisposition and other organ dysfunction
• Myeloid neoplasms with germ line GATA2 mutation • Myeloid neoplasms associated with bone marrow failure syndromes • Juvenile myelomonocytic leukemia associated with neurofibromatosis, Noonan syndrome, or Noonan syndrome-like disorders • Myeloid neoplasms associated with Noonan syndrome • Myeloid neoplasms associated with Down syndrome[1]

1. Lymphoid neoplasms also reported.

1.2.3 Mutational landscape of AML

Different cancer types carry various numbers of mutations but among cancers, AML is one of the types of cancer with the lowest number of mutations in comparison to other adult cancers (Figure 6) (Alexandrov, Nik-Zainal et al. 2013, Lawrence, Stojanov et al. 2013). Genetic alterations initially described as karyotypic abnormalities are found in roughly 50% of AML patients by cytogenetic analysis (Dohner, Estey et al. 2017, Medinger, Lengerke et al. 2016). However, almost half of AML cases do not have any karyotypic abnormalities, and hence are described as cytogenetically normal AML (CN-AML) patients. In CN-AML samples, the mean number of mutations in coding sites, splice sites and RNA genes are around 13 (ranging between 0-51) including eight passenger mutations and five driver mutations (Cancer Genome Atlas Research, Ley et al. 2013). So far, numerous mutations have been identified in AML patients, the most frequent of which are depicted in Figure 7. Below, some of the most frequent mutations which cause AML or are important in the development or maintenance of leukemic cells are described.

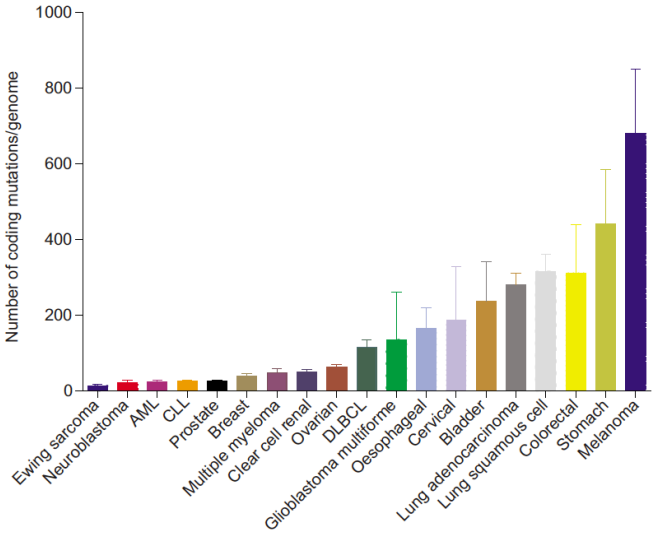

Figure 6. The mean number of mutations per genome in various cancer types. Figure reprinted with permission from the publisher (Grove and Vassiliou 2014).

Nucleophosmin 1 (*NPM1*) mutations

The nucleolar protein nucleophosmin 1 plays various cellular functions including protein chaperoning, centrosome duplication, DNA repair, cell proliferation and apoptosis. The most common mutation in AML patients is *NPM1* mutations with a frequency of 25-30% in all AML cases and more specifically 45-64% in CN-AML (Falini, Nicoletti et al. 2007, Schnittger, Schoch et al. 2005). The exact mechanism of *NPM1* in leukemogenesis has not yet been clarified. Since *NPM1* has multiple functions, it is thought that *NPM1* mutations drive leukemia through a combination of cellular processes. Loss of function due to *NPM1* mutations causes uncontrolled centrosome duplication and also promotes myeloproliferation and susceptibility to oncogenic transformation. Moreover, it induces ubiquitination and degradation of TP53 by MDM2. On the other hand, gain of function in cytoplasmic NPM1 inhibits caspase enzymes which blocks apoptosis, induces expression of *Myc* and increases the cell proliferation rate (Heath, Chan et al. 2017).

NPM1 mutations in AML cases are frequent in the nucleolar localization signal located at the N-terminal and cause abnormal expression of cytoplasmic NPM1 protein rather than in the nucleus. Furthermore, *NPM1* mutations are correlated with *DNMT3A*, *FLT3-ITD* and +8 (40%), *FLT3-TKD* (10-15%) and *IDM* mutations (25%) (Marcucci, Haferlach et al. 2011, Marcucci, Metzeler et al. 2012).

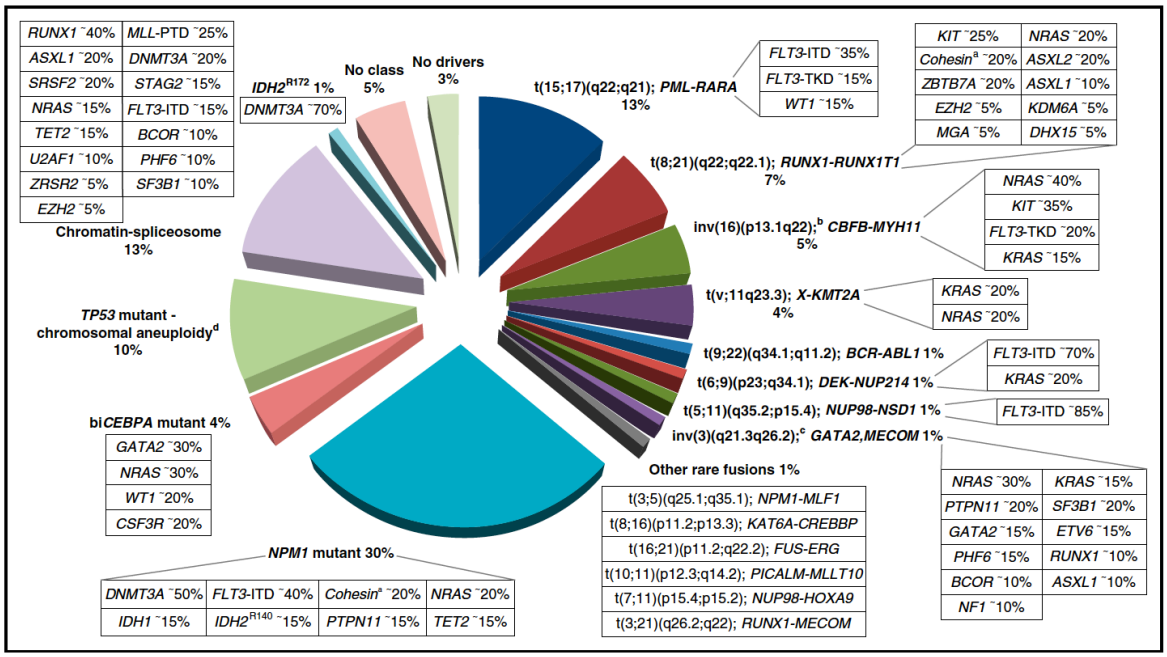

Figure 7. Common gene mutations in adult AML patient up to 65 years old. For each gene, the frequency of correlation with other mutation s are shown in the boxes. a indicates cohesin genes including RAD21 (~10%), SMC1A (~5%), and SMC3 (~5%). b inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11. c inv (3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1). d TP53 mutations are found in ;45%, and complex karyotypes in ;70% of this class. Figure reprinted with permission from the publisher (Dohner, Estey et al. 2017).

Fms-like tyrosine kinase 3 (*FLT3*) mutations

FLT3, a tyrosine kinase receptor for the FLT3 ligand was first found with high expression pattern in HSCs and plays an important role in the survival and proliferation of hematopoietic cells (Gilliland and Griffin 2002, Maroc, Rottapel et al. 1993). The most frequent mutations in *FLT3* are in the second tyrosine kinase domain (TKD) or the internal tandem duplication (ITD) in the juxta-membrane domain, which are identified in 20% of all AML patients and 30-45% of CN-AML cases (Kelly, Liu et al. 2002). Gain of function in *FLT3* causes constitutive phosphorylation of some downstream proteins including GAB1, GAB2, SHP-2, AKT, FOXO3a, CBL, STAT5a, ERK1/2, GRB2, SHC, VAV, LYN, GAP, p90RSK, BAD, C/EBPα, and SHI through PI-3 kinase/AKT, RAS/ MAPK, and STAT5 pathways in leukemic cells (Small 2006).

Isocitrate dehydrogenase (*IDH*) mutations

These enzymes catalyze the oxidative decarboxylation of isocitrate to 2-oxoglutrate. *IDH1* and *IDH2* mutations were identified in 15-20% of all AML patients and 25-30% of CN-AML cases, most often altering the highly conserved arginine residue at codon 132 (R132) of *IDH1*

and at codons R140 and R172 of *IDH2* (Marcucci, Maharry et al. 2010, Patel, Gonen et al. 2012). Moreover, *IDH1* and *IDH2* mutations are also detected more in older AML patients (Paschka, Schlenk et al. 2010).

Mutations of these enzymes in AML, is a gain of function, giving them a new ability to covert α-ketoglutarate into 2-hydroxyglutarate (2-GH). High levels of 2-GH inhibit TET enzymes, leading to accumulation of histone methylation, subsequently blocking myeloblast differentiation, and increasing stem cell marker expression, suggesting a pro-leukemic effect of *IDH1/2* mutations (Figueroa, Abdel-Wahab et al. 2010, Lu, Ward et al. 2012, Ward, Patel et al. 2010).

DNA methyltransferase 3A (*DNMT3A*) mutations

As previously mentioned, the DNA methyltransferase DNMT3A is involved in *de novo* methylation. *DNMT3A* mutations induce global hypomethylation, which is associated with genome instability, and hypomethylation in promoter regions of *HOX* and *MEIS1* genes, which are associated with transcriptional activation (Ferreira, Heyn et al. 2016). *DNMT3A* mutations occur in 18-22% of AML patients and commonly presents as a missense mutation located on R882 compared to other codons in the *DNMT3A* gene (non-R882) (Ley, Ding et al. 2010). *DNMT3A* mutations were detected in the early stages of AML evolution as pre-leukemic mutations and persist during remission (Shlush, Zandi et al. 2014). Yet, a large study of 1700 AML patients revealed that there is no significant correlation between *DNMT3A* mutations and survival (Gaidzik, Schlenk et al. 2013).

Tet methylcytosine dioxygenase 2 (*TET2*) mutations

TET2 is a methylcytosine dioxygenase that converts 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), which plays key role in DNA demethylation. The most common type of *TET2* mutation is loss of function which was found in 9-23% of AML cases (Chou, Chou et al. 2011), however, the prognostic effects of *TET2* mutations remain controversial (Gaidzik, Paschka et al. 2012, Metzeler, Maharry et al. 2011).

Tet2 mutations in mice are associated with DNA hypermethylation, altered gene expression patterns and subsequent increased self-renewal of HSCs and myeloid expansion (Cimmino, Dolgalev et al. 2017).

Runt-related transcription factor (*RUNX1*) mutations

RUNX1, also known as AML1 protein or core-binding factor subunit α-2 (CBFA2), is a TF with an essential role in normal hematopoiesis (Meyers, Downing et al. 1993). Translocation of *RUNX1* at chromosome 21 with the *ETO* gene on chromosome 8 creates the fusion protein RUNX1-ETO (RUNX1-RUNX1T1) which is frequently found in AML cases (Tang, Hou et al. 2009). The fusion protein RUNX1-ETO suppresses the function of normal RUNX1 in hematopoiesis which induces repression of myeloid-specific genes, causing a block in myeloid cell maturation (Ichikawa, Yoshimi et al. 2013). Moreover, other mutations in *RUNX1* were found in 5-13% of AML cases and were frequently associated with trisomy 13, 21, old CN-AML patients, absence of *NPM1* and poor outcome (Marcucci, Haferlach et al. 2011, Mendler, Maharry et al. 2012, Tang, Hou et al. 2009).

CCAAT enhancer binding protein A (*CEBPA*) mutations

CEBPA is a TF which regulates the expression of important genes required for hematopoiesis (Koschmieder, Halmos et al. 2009). *CEBPA* mutations were detected in 6-10% of all AML patients and 15-19% of CN-AML, generally associated with del (9q) (Mrozek, Marcucci et al. 2007). Conditional deletion of *Cebpa* in adult mice blocks CMP to GMP transition and consequently leads to an accumulation of myeloblasts in the BM (Zhang, Iwasaki-Arai et al. 2004). In most AML cases with *CEBPA* mutations, patients often simultaneously carry two mutations (double mutations) commonly known as biallelic in the N-terminal and bZIP regions. Double biallelic mutations, but not single *CEBPA* mutations are associated with favorable overall and higher complete response (CR) (Fasan, Haferlach et al. 2014, Wouters, Lowenberg et al. 2009).

Mixed Lineage Leukemia (*MLL1*) mutations

MLL1 (Lysine-specific methyltransferase 2A or KMT2Aencodes) is a histone methyltransferase enzyme located on chromosome 11 (11q23). In AML, more than 80 different protein partners were found to generate a fusion oncogene through translocation with *MLL* (Meyer, Hofmann et al. 2013). Leukemia with *MLL* translocations manifest as either acute lymphoid, myeloid or mixed phenotype acute leukemia (MPAL), which are detected in 10% of all leukemia cases (Muntean and Hess 2012). But the incidence of *MLL* rearrangements is 70-80% in infant leukemia patients (Mann, Attarbaschi et al. 2010).

Also, *MLL* rearrangements are common in therapy-related leukemia, typically in patients treated with topoisomerase II inhibitors (e.g., ectoposide, doxorubicin) (Super, McCabe et al. 1993). Translocation of *MLL* is common in AML and it is correlated with poor prognosis. The frequencies of the main partners involved in fusion of *MLL* (*AF4*, *AF9*, *ENL*, *AF10*, *ELL* and *AF6*) in AML are shown in Figure 8. In addition to *MLL* translocations, partial tandem duplication (PTD) of *MLL* is most commonly identified in *de novo* CN-AML and in AML patients with trisomy 11 (Caligiuri, Schichman et al. 1994, Caligiuri, Strout et al. 1998).

MLL rearrangements cause upregulation of several *HOX* genes (including *HOXA5*, *HOXA9*, and *HOXA10*) and *MEIS1*, which contribute to the growth advantage and maintenance of self-renewal properties of AML cells (Li, Luo et al. 2009).

Additional sex comb-like 1 (*ASXL1*) mutations

ASXL1 is a chromatin-binding protein involved in the epigenetic regulation of cells, yet the clear function of ASXL1 in hematopoiesis is not fully understood. *ASXL1* mutations occur in 5-11% of all AML patients and the mutation rate is five times higher in patients over 60 compared to those under 60 years old (Metzeler, Becker et al. 2011). *ASXL1* mutations cause loss of H3K27me3 mediated by the PRC2 complex and upregulated expression of *HOXA* genes. *Asxl1* mutations in combination with NRasG12D expression promotes myeloid leukemogenesis *in vivo (Abdel-Wahab, Adli et al. 2012)*.

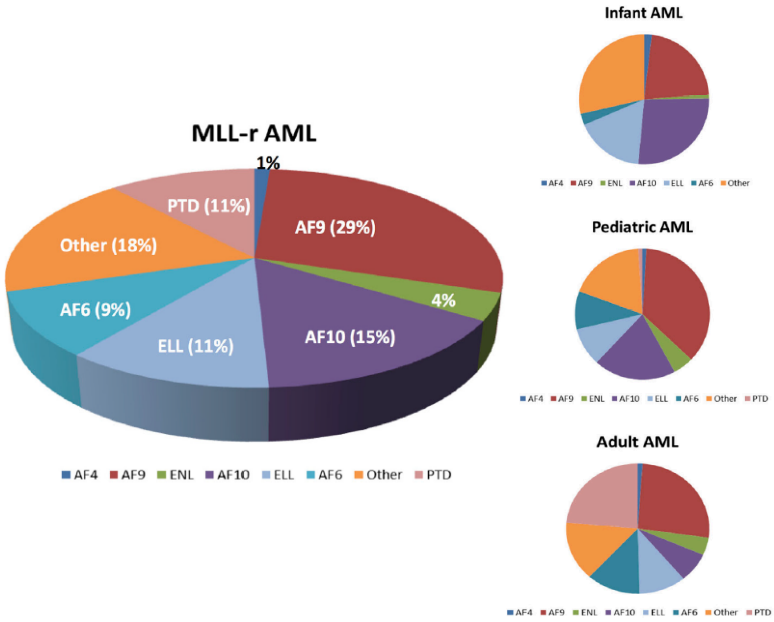

Figure 8. The Frequency of MLL-rearranged AML with main seven fusion partners. The right pie charts show the breakdown of the relative frequencies of MLL fusion partners based on age group. Figure reprinted with permission from the publisher (Winters and Bernt 2017).

***Tumor protein p53* (*TP53*) mutations**

TP53 regulates quiescence and self-renewal of HSCs, and so *p53* mutations promote HSPC proliferation, leading to an accumulation of DNA damage and ultimately transformation of HSCs into pre-leukemic cells (Prokocimer, Molchadsky et al. 2017). Mutations of *TP53* are reported in 8-14% of AML cases and *TP53* alterations are most often associated with a complex karyotype, poor prognosis and resistance to chemotherapy (Haferlach, Dicker et al. 2008, Rucker, Schlenk et al. 2012).

***KIT* mutations**

KIT tyrosine kinase is a transmembrane receptor for mast cell growth factor (MGF) and critical for normal hematopoiesis (Sattler and Salgia 2004). The *KIT* mutation frequency is less than 5% in all AML patients, but identified in 22-29% of AML cases with CBF mutations (Paschka, Marcucci et al. 2006). Moreover, overexpression of *KIT* is identified in 60-80% of AML patients (Kindler, Breitenbuecher et al. 2004). *KIT* mutations are associated with a high relapse rate and shorter overall survival (Boissel, Leroy et al. 2006).

KIT mutations trigger constitutive KIT kinase activation, promoting downstream signaling pathways which contribute to factor-independent growth of hematopoietic cells and induce leukemogenesis (Lennartsson, Jelacic et al. 2005).

1.2.4 Prognosis factors

A crucial component of AML treatment is an accurate assessment of AML prognosis. Prognostic factors can provide information for physicians to decide on a treatment strategy between standard or increased intensity treatment, consolidation chemotherapy or transplantation. Prognostic factors can be divided in two categories: patient-related, such as age and performance status and disease-associated factors including white-cell count, cytotoxic therapy for another disorder or prior myelodysplastic syndrome, cytogenetic and molecular aberrations (Bullinger, Dohner et al. 2017, Medinger, Lengerke et al. 2016, Meyer and Levine 2014).

In 2010, the European LeukemiaNet (ELN) proposed a standardized classification of risk stratifications in adult AML patients according to the correlation of cytogenetic and molecular genetics with clinical data (Dohner, Estey et al. 2010). In the updated 2017 classification, patients are divided into three risk groups: favorable, intermediate and adverse.

(Table 6) (Dohner, Estey et al. 2017). Recently, the new techniques such as FISH, polymerase chain reaction (PCR) and gene sequencing have provided tools to enable a more comprehensive view of AML prognosis.

In abnormal karyotype patients, alterations of chromosome 5, 7, 11q23 and more than three chromosomal abnormalities are associated with poor prognosis and reduced survival, whilst t(8;21)(q22;q22), t(15;17)(q22;q12) and inv(16)(p13.1;q22) abnormalities are associated with longer survival (Dohner, Weisdorf et al. 2015, Medinger and Passweg 2017).

KIT mutations in patients with t(8;21) translocation increase the risk of relapse and decreased overall survival (Estey 2014, Qin, Zhu et al. 2014). The presence of *TP53* or *DNMT3A* mutations are associated with poor prognosis (Kihara, Nagata et al. 2014, Shivarov, Gueorguieva et al. 2013). In CN-AML, partial tandem duplication of MLL is associated with a worse prognosis (Patel, Gonen et al. 2012).

Several studies have shown that *FLT3-ITD* mutations in older CN-AML patients (>60 years old) are associated with poor outcome (Patel, Gonen et al. 2012, Port, Bottcher et al. 2014). ITD of *FLT3* is associated with a poor prognosis and high relapse rate (Kelly, Liu et al. 2002, Meyer and Levine 2014, Saultz and Garzon 2016), constitutively activates tyrosine kinase and subsequently activates RAS, MAPK and STAT5 pathways and induces cell proliferation (Gale, Green et al. 2008, Kayser, Schlenk et al. 2009, Kelly, Liu et al. 2002).

Table 6. Risk assessment of AML according European LeukaemiaNET (ELN) 2017. Table adapted with permission from the publisher (Medinger and Passweg 2017).

Risk category	Genetic abnormality
Favorable	• t(8;21)(q22;q221); RUNX1-RUNX1T1 • Inv(16)(p131q22) or t(16;16)(p131;q22); CBFB-MYH11 • Mutated NMP1 without FLT3-ITD or with FLT3-ITD^{low} (allelic ratio <0.5) • Biallelic mutated CEBPA
Intermediate	• Mutated NPM1 and FLT3-ITD^{high} (allelic ratio ≥0.5) • Wild-type NPM1 without FLT3-ITD or with FLT3-ITD^{low} (without adverse-risk genetic lesions) • t(9;11)(p213;q233); MLLT3-KMT2A • Cytogenetic abnormalities not classified as favorable or adverse
Adverse	• t(6;9)(p23;q341); DEK-NUP214 • t(v;11q233); KMT2A rearranged • t(9;22)(q341;q112); BCR-ABL1 • inv(3)(q213q262) or t(3;3)(q213;q262); GATA2, MECOM (EVI1) • -5 or del (5q); -7; -17/abn(17p) • Complex karyotype (three or more unrelated chromosome abnormalities, monosomal karyotype (defined by the presence of 1 single monosomy) • Wild-type NPM1 and FLT3-ITD^{high} • Mutated RUNX1 • Mutated ASXL1 • Mutated TP53

NMP1 mutations are associated with sensitivity to intensive chemotherapy, which may result in improved outcomes in both young and older patients (Dohner, Estey et al. 2017, Dohner, Schlenk et al. 2005).

As a result, these three markers *NPM1* mutation, *CEBPA* mutation, and *FLT3* ITD are currently used in clinical practice (Dohner, Estey et al. 2010). Therefore, deep genetic analyses of AML patient samples are critical for prognostic classification and treatment plan strategies, however the ability to predict resistance to treatment and relapse remains limited (Walter, Othus et al. 2015).

1.2.5 The bone marrow niche of AML

Similar to normal HSCs, leukemic cells also need a proper niche in which to reside and expand. Recent studies have confirmed that the BM niche supports development and expansion of hematopoietic malignancies. Massive alterations of the BM microenvironment with age, in addition to mutation accumulation, contribute to leukemogenesis. For example, activation of the osteoblastic cell-specific parathyroid hormone (PTH) receptor enhanced frequency of LSCs in the *MLL-AF9* AML mouse model (Krause, Fulzele et al. 2013).

Interestingly, there is also evidence that alterations to the BM niche can induce AML. For instance, deletion of *Dicer1* or *Sbds* in osteoprogenitors induced myelodysplasia and predisposition to AML (Raaijmakers, Mukherjee et al. 2010). Additionally, activating mutation of ß-catenin in osteoblast cells induces high activation of Notch signaling in progenitor cells, resulting in an altered differentiation of myeloid and lymphoid cells, and ultimately inducing AML (Kode, Manavalan et al. 2014). Altogether, these studies indicate that alterations to the BM niche can provide appropriate conditions which promote hematopoietic malignancies.

Further studies have demonstrated that the BM niche is altered during development of CML-blast crisis resembling AML by induction signals from the leukemic cells. It has been shown that leukemic cells in direct contact with MSCs secrete TPO and CCL3 that stimulate MSCs to overproduce functionally altered osteolineage cells which support LICs (Schepers, Pietras et al. 2013).

In summary, it seems that the BM niche and leukemic cells have mutual cross-talk during initiation, development and AML relapse.

1.2.6 Clonal hematopoiesis and AML evolution

Since HSCs divide during the whole human life, they may acquire random mutations with time which accumulate and are passed on to the next generation of cells. Although most mutations in HSCs are benign, mutations that induce enhanced self-renewal capacity or proliferation of HSCs and/or reduce apoptosis, cause clonal advantage.

The two-hit model was first proposed by Gilliland and Griffin (Gilliland and Griffin 2002) for clonal hematopoiesis (CH) and subsequently leukemogenesis. In this model two classes of mutations are required. Class I mutations contribute to the activation of proliferation pathways or to survival advantage such as *FLT3-ITD*, *K/N-RAS*, *TP53* and *c.KIT*. Whereas class II mutations impair normal differentiation of hematopoietic cells such as *NPM1*, *CEBPA*, *PML/RARα* and *MLL* fusion genes. In addition to these two classes of mutations, there are some mutations of epigenetic factors such as *DNMT3A*, *TET2*, *IDH1* and *IDH2* which effect both proliferation and differentiation, and so can be classified in to a third group (De Kouchkovsky and Abdul-Hay 2016).

Additionally, other mechanisms such as the immune system, BM niche alterations and selective pressure on somatic variants (aging, chemotherapy and exogenous stress such as smoking) also influence CH (Bowman, Busque et al. 2018). The most common mutations present in CH are in epigenetic factors like *TET2*, *DNMT3A* and *ASXL1* and splicing factors such as *SF3B1*, *SRSF2* and *U2AF1* (Bowman, Busque et al. 2018). These mutations in CH prime them to develop hematopoietic malignancies.

Mutations that contribute to cancer initiation and growth are called driver mutations, whilst those that do not affect cancer growth are termed passenger mutation. However, the classification of cancer depends on its microenvironment. For example, certain AML mutations can confer drug resistance so that during cancer treatment sub-clones carrying these mutations can survive and expand, whereas in the absence of treatment these mutations might have a growth disadvantage effect (Grove and Vassiliou 2014). On the other hand, some passenger mutations may be selected in a specific microenvironment condition and induce drug resistance or growth advantage.

Cancer evolution similar to Darwinian natural selection was first suggested by Peter Nowell (Nowell 1976). Emerging data from AML patient samples using new technologies, particularly NGS, are providing a better view of mutational frequencies, clonal evolution of the disease, sub-clonal patterns and into the epigenetic landscape of AML. The result from a clonality analysis of 50 whole genome sequences and 150 whole-exomes sequences of AML patient samples revealed that the majority of AML cases are clonally heterogeneous with a

founding clone and at least one sub-clone at the diagnosis stage (Cancer Genome Atlas Research, Ley et al. 2013). Importantly, two major clonal evolution patterns during relapse were determined: either a founding clone or a surviving sub-clone acquired additional mutations most likely contributed to drug resistance and expanded at relapse stage (Ding, Ley et al. 2012).

New findings from NGS of pre-leukemic stem cells and clonal evolution of AML revealed that pre-leukemic HSCs already contain recurrent common mutations in epigenetic regulators, such as *DNMT3A*, *ASXL1*, *IDH1*, *IDH2*, and *TET2*. They are therefore thought to occur in the early stage of AML evolution (Corces-Zimmerman, Hong et al. 2014, Kronke, Bullinger et al. 2013, Shlush, Zandi et al. 2014). Moreover, the frequency of somatic mutations (*DNMT3A*, *TET2*, and *ASXL1*) were rare in individuals under 40 years old but increased with age and the presence of these mutations was associated with a higher risk of hematologic cancers (Busque, Patel et al. 2012, Genovese, Kahler et al. 2014, Jaiswal, Fontanillas et al. 2014). Other somatic mutations which are known in AML are *FTL3*, *NPM1*, *WT1*, *RUNX1*, *RTRP*, *PHF6* and *ETV6* (Zhang, Lv et al. 2016).

1.2.7 Leukemic stem cells

A small population of cells amongst AML blast cells, have limitless self-renewal capacity and the ability to propagate and go on to develop AML. These cells are known as leukemic stem cells (LSCs) or leukemia initiating cells (LICs). LICs are a rare population (roughly 1 per 10^6 bulk) in many AML patients capable of initiating leukemia in immunodeficient mice and maintaining long-term in serial transplanted mice with self-renewing capacity. There is some evidence showing that LICs are not only originated from HSCs, but certain mutations in differentiated cells may also transform them in to LSCs (Cozzio, Passegue et al. 2003, Huntly, Shigematsu et al. 2004).

The critical step of LIC detection is to define markers which are associated with functional properties of LICs. LICs usually exist in the $CD34^+CD38^-$ fraction of AML cells in patients (Lapidot, Sirard et al. 1994). However in more than 50% of samples LICs reside in $CD34^+CD38^+$ or even the $CD34^-$ population (Kreso and Dick 2014). Further experiments revealed in 80% of AML patients LSCs exist in both fractions; LMPP-like (lymphoid-primed multipotent progenitors) LSCs ($Lin^-CD34^+CD38^-CD90^-CD45RA^+$) and GMP-like LSCs ($Lin^-CD34^+CD38^+CD123^+CD45RA^+$). Interestingly, LMPP-like LSCs could give rise to GMP-like LSCs but not the converse (Goardon, Marchi et al. 2011).

From a clinical perspective, to achieve long-term complete remission (CR), it is critical to eliminate LICs. However, there are several obstacles to target LICs with conventional therapies. First, there is no standard method to characterize LICs which demonstrate their properties. Secondly, LICs are a rare population and quiescent so it is difficult to eliminate them with current drugs which target proliferative bulk cells. Thirdly, LICs are usually resistant to apoptosis and they highly express channels for drug efflux (Thomas and Majeti 2017). In addition, recent studies have shown heterogeneity in LICs both between and within individual patients (Eppert, Takenaka et al. 2011, Goardon, Marchi et al. 2011). Thus, it is needed to identify new targets against LICs to develop more effective therapies.

1.2.8 AML treatment

Current therapy

The common therapeutic approach in AML patients not much has changed since 1970 (Dohner, Estey et al. 2010). The first line of treatment is to assess whether intensive chemotherapy is suitable for a AML patient. After intensive therapy, post-induction therapy is important to achieve CR. Two types of post-induction strategies are: conventional chemotherapy and hematopoietic cell transplantation.

Induction therapy

The aim of induction therapy is to diminish AML cells and achieve CR. The standard induction therapy is chemotherapy. Intensive induction therapy is applicable to young adult AML patients under 60 years old and older patients specifically with *NPM1* mutations and CBF leukemia (Saultz and Garzon 2016). The standard chemotherapy protocol is the "7+3" regimen with a combination of continuous infusion of cytarabine (100 mg/m^2 daily for one week) and an anthracycline (either daunorubicin 60-90 mg/m^2 or idarubicin 10-12 mg/m^2 on days 1, 2 and 3). The current conventional therapies for AML patients are summarized in Table 7.

The CR rate is 65-73% in adult patients younger than 60 years and 38-62% in patients over 60 years old (Estey and Dohner 2006, Fernandez, Sun et al. 2009, Lowenberg, Ossenkoppele et al. 2009). CR is defined as (1) <5% blasts in a BM aspirate sample with marrow spicules (no blasts with Auer rods or persistence of extramedullary disease), (2) absolute neutrophil count (ANC) >1000/μl, and (3) platelets ≥100,000/μl (Cheson, Bennett et al. 2003).

Consolidation (post-induction) therapy

The aim of consolidation strategies is to eradicate minimal residual disease (MRD), prevent relapse and ideally to achieve a complete cure with either chemotherapy or transplantation. MRD can be monitored by using molecular based approaches such as real-time quantitative PCR (RT-qPCR) or target gene sequencing to detect genetic targets specific to AML cells and/or multi-parameter flow cytometry (MFC) to read-out leukemic cells based on their immune phenotype (Grimwade and Freeman 2014).

In consolidation chemotherapy, intermediate dose of cytarabine (1-1.5g/m^2) is used twice daily on days 1, 3 and 5 in three or four cycles in adults younger than 60 years old, where the cure rate is 60-70% (Byrd, Mrozek et al. 2002). However, in patients older than 60 years, the cure rate is dismal, between 10-15%, therefore new strategies of maintenance therapies need to be investigated (Dohner, Weisdorf et al. 2015).

Hematopoietic stem cell transplantation is a treatment strategy for patients who are unlikely respond to the conventional induction therapy and to extend CR. Prior to transplantation, chemoradiotherapy is chosen to deplete leukemic cells, suppress the immune system, and to

Table 7. Summary of current conventional care of AML patients. Table adapted with permission from the publisher (Dohner, Weisdorf et al. 2015).

Type of therapy	Age	Regimen
Induction therapy	Patients 16–60 yr	3 days of an intravenous anthracycline (daunorubicin 60 mg/m^2; idarubicin 10-12 mg/m^2; mitoxantrone 10-12 mg/m^2) and 7 days of continuous-infusion cytarabine (100-200 mg/m^2) ("3+7" induction)
	Patients >60 yr	For patients with favorable-risk and intermediate-risk cytogenetic findings and no coexisting conditions, induction therapy is the same as that in younger patients, and dose reduction may be considered for individual patients
Consolidation therapy	Patients 16–60 yr	Patients with favorable genetic risk (according to ELN) should Receive 2-4 cycles of intermediate-dose cytarabine (1-1.5 g/m^2 intravenously, usually administered every 12 hr over 3 days, or 1-1.5 g/m^2 intravenously on days 1-6); for patients with intermediate-I, intermediate-II, or adverse risk, allogeneic hematopoietic-cell transplantation should be strongly considered; if not possible, consolidation therapy should be administered as above; combination chemotherapy (e.g., mitoxantrone-cytarabine) may be superior in patients with adverse-risk AML
	Patients >60 yr	Patients with favorable ELN genetic risk (less common) and no coexisting conditions should receive 2-3 cycles of intermediatedose cytarabine (0.5-1 g/m^2 intravenously, every 12 hr on days 1-3, or 0.5-1 g/m^2 intravenously, on days 1-6)

provide space for donor cells in in the BM. The most common regimen for chemoradiotherapy is fludarabine in combination with cyclophosphamide or other alkylating reagents (such as melphalan and busulfan) and total-body irradiation. The key dilemma after transplantation is graft-versus-host-disease (GVHD), in which donor immune cells recognize the host cells as foreign and attack the recipient's organs. Therefore, accurate HLA matching is required to decrease the risk of GVHD.

Despite new techniques such as NGS to predict prognosis and to improve treatment strategy selection, the heterogeneity of AML and the varied genetic landscape of sub-clones make it difficult to estimate the risk of relapse.

Novel treatment strategies

Since AML is a heterogeneous disease within patients and even within sub-clones, the standard chemotherapy strategy is not the best option for all patients and given that chemotherapy does not specifically target leukemic cells, it is known to cause severe side effects. Personalized medicine (also known as precision medicine) is the new goal in the cancer field, which refers to individual patient based-strategies, where their genetic background, prognosis and predicted response and treatment strategies are tailored to each individual patient. However, it is demanding to design new drugs or inhibitors specifically targeting driver genes in cancer cells for treatment based on the mutational background to improve cure rate, increase survival rate and prevent relapse. Here, new inhibitors and treatment strategies in AML are briefly described.

FLT3 Inhibitors

The first generation of multiple kinase inhibitors such as midostaurin, lestaurtinib, tandutinib, sunitinib and sorafenib, have recently shown transient reduction of leukemic cells and increased toxicity (Sudhindra and Smith 2014). The second generation of *FLT3* inhibitors including quizartinib and crenolanib are being used in the clinical phase and have better potency with less side effects. However, drug resistance is a main obstacle when using single inhibitor for FLT3 mutations. Recently, the drug Midostaurin was approved by the FDA to be used in combination with daunorubicin and cytarabine for *FTL3* mutation positive AML patients. Additionally, G-749 and ASP2215, as novel *FLT3* inhibitors with a decreased risk of drug resistance, have recently been used in clinical trials (Lee, Kim et al. 2014).

IDH Inhibitors

In 20% of AML samples *IDH-1* or *IDH-2* have gain of function mutations (Cancer Genome Atlas Research, Ley et al. 2013). There are some *IDH1* and *IDH2* inhibitors currently in phase I and II clinical trials, such as NCT02381886, NCT0195498 and NCT02074839, AG-221 and AG-120, CB-839, Erwinaze (Fathi, Wander et al. 2015, Medinger and Passweg 2017). The response rates in relapsed patients to AG-221 and AG-120 was 40% and 31% respectively.

HDAC inhibitors

Epigenetic modifiers HDACs catalyze deacetylation of histones and involve in gene silencing. Mutations or dysregulations of many acetyltransferases and deacetylases have been identified in leukemia including p300, CBP, AML1. Therefore, HDAC inhibitors including varinostat, mocetinostat and SAHA appeared as an attractive therapeutic strategy for AML. Since the response rate in monotherapy studies using single HDAC inhibitors were low (13-17%), the current trial strategies focus on combination of HDAC inhibitors with chemotherapy or other epigenetic inhibitors (Saygin and Carraway 2017).

STAT inhibitors

Deregulation of STAT signaling in AML is associated with an increased expansion, apoptosis block and abnormal differentiation of leukemic cells. Constitutive activation of STAT3 and STAT5 either alone or together has been reported in AML patients (Bar-Natan, Nelson et al. 2012). Furthermore, *STAT3* tyrosine phosphorylation is associated with a worse prognosis and was found to be upregulated in 50% of AML patients (De Kouchkovsky and Abdul-Hay 2016). Moreover, overexpression of STAT3 signaling contributes to *FLT3* inhibitor resistance in AML cells (Zhou, Bi et al. 2009). Therefore, *STAT3* inhibitors such as OPB-31121 could be used for patients who are being treated with *FLT3* inhibitors.

MLL inhibitors

There are many approaches to target MLL fusion proteins in AML patients with MLL translocations. One target is Disruptor of telomeric silencing 1-like (DOT1L), a histone methyltransferase, which is recruited by most of the MLL fusion proteins. Abnormal interaction of MLL oncogene and DOT1L results in methylation of H3K79 and activation of

MLL downstream target genes including *HOXA9* and *MEIS1* (Kavanagh, Murphy et al. 2017). Other inhibitors against Menin, Polycomb proteins, LSD1 and Bromodomain proteins can be used to target MLL-rearranged leukemias (Winters and Bernt 2017).

Nuclear Exporter Inhibitors

Chromosome region maintenance 1 (CRM1) is a nuclear exporter protein which is responsible for exporting several tumor suppressor proteins such as P53, P21, P73, FOXO1, RB1 and NMP1 (Fukuda, Asano et al. 1997). Overexpression of CRM1 causes exorbitant transportation of tumor suppressor proteins from the nucleus to the cytoplasm, which is associated with poor prognosis and decreased survival (Kojima, Kornblau et al. 2013). Selinexor as a CRM1 inhibitor is in clinical trials to be used as a drug for AML patients with high CRM1 expression levels (Etchin, Sun et al. 2013, Ranganathan, Yu et al. 2012).

Clofarabine

Clofarabine is a second-generation purine nucleoside analogue FDA-approved for treating relapsed or refractory childhood ALL patients. Clofarabine enters cells via passive or active transport and is transformed to its active triphosphate form by kinases. Clofarabine inhibits both DNA polymerase and ribonucleotide reductase, which in turn inhibits DNA replication as well as RNA transcription (Tiley and Claxton 2013). Treatment of older AML patients with Clofarabine resulted in a 40% overall response rate (Burnett, Russell et al. 2013, Kantarjian, Erba et al. 2010). Combination of Clofarabine with a low dose of cytarabine significantly increased the CR rate from 31% to 63% in patients over the age of 60 (Faderl, Ravandi et al. 2008).

Immune and Cell Therapies

A new strategy of induction therapy is immune therapy using an anti-CD33 antibody, which in testing showed a similar response rate compared to chemotherapy treatment but reduced risk of relapse and increased survival. The transmembrane receptor CD33 is expressed on cells of myeloid origin but not on normal HSCs. Widespread expression of CD33 in AML cells promoted the idea of targeting CD33 to treat AML patients (Appelbaum and Bernstein 2017). A humanized anti-CD33 monoclonal antibody (Gemtuzumab ozogamicin) conjugated with the cytotoxic agent calicheamicin was tested in five randomized trials and approved by

the FDA in 2000 (Hills, Castaigne et al. 2014). Although further clinical trials in 637 patients failed to show improvements in CR or overall survival (Petersdorf, Kopecky et al. 2013), recent trials showed a benefit in survival and relapse rates in old AML patients not eligible for intensive chemotherapy and also in patients with an intermediate to favorable cytogenetic-risk pattern (Amadori, Suciu et al. 2016).

Another new cell therapy strategy is to use engineered T cells called chimeric antigen receptor (CAR)-transduced T cells (CARTs) to express antigen receptors against specific cell surface antigens in target AML cells. One type of CARTs in preclinical trials is against CD123, which is expressed on the majority of AML cells (Gill, Tasian et al. 2014). CD33-specific CART cells showed improved survival in a xenograft AML mouse models (Kenderian, Ruella et al. 2015). New therapeutic agents in ongoing clinical trials for AML treatment are listed in Table 8.

Table 8. Summary of new therapeutic agents in ongoing clinical trials. Table adapted with permission from the publisher (Yu and Zheng 2017).

Agent	Name	Target	Phase of testing
Molecular antibody	Gemtuzumab ozogamicin	CD33	I, II, III
	Vadastuximab talirine (SGN-CD33A)	CD33	I, II
	AMG 330	CD33	I
	HuM195	CD33	I, II
	Yttrium Y 90 anti-CD45 monoclonal antibody BC8 (90Y-BC8)	CD45	NA
	KB004	EphA3	I, II
	Ipilimumab	CTLA-4	I
	Brentuximab	CD30	I, II
	Ulocuplumab	CXCR4	I
FLT3 inhibitors	Lestaurtinib	FLT3	I, II
	Midostaurin	FLT3	I, II, III
	Sorafenib	FLT3	I, II, IV
	Quizartinib	FLT3	I, II, III
	Crenolanib	FLT3	I, II, III
	Gilteritinib	FLT3	II, III
	Pexidartinib (PLX3397)	FLT3	I, II
AURK inhibitors	Alisertib	AURKA	I, II
mTOR kinase inhibitors	Sirolimus	mTOR	I, II
	Temsirolimus	mTOR	I, II
	Everolimus	mTOR	I, II
Epigenetic agents	Decitabine	Methyltransferase	I, II, III
	Azacitidine	Methyltransferase	I, II
	Vorinostat	Histone acetylase	I, II
	Panobinostat	Histone acetylase	I, II
CAR-T cell therapy	CAR-T33	CD33	I, II
	CAR-T123	CD123	I, II

1.2.9 Childhood AML

Among all AML cases, 6% of them develop in childhood (Dores, Devesa et al. 2012) and 20% of pediatric leukemic cases are AML (Yu and Zheng 2017). The survival rate of AML in patients under 20 years old have improved during the last 40 years from 20% in 1980 to 70% in 2012 of AML cases, but the clinical outcomes of childhood AML in many subtypes remains low (Gamis, Alonzo et al. 2013, Rubnitz and Inaba 2012, Rubnitz, Inaba et al. 2014).

Certain mutations common in adult AML are rare in childhood AML and vice versa, suggesting differing mechanisms and pathogenesis of AML in adults and children. The main molecular aberrations in childhood AML are depicted in Figure 9. The major differences between childhood AML compared to adult AML are a significantly higher frequency of *MLL*-related leukemia, particularly in patients under two years old, and a lower incidence of cytogenetic abnormalities (Cooper, Franklin et al. 2012). Therefore, the role of molecular risk factors such as *FLT3-ITD*, *IDH1* and *DNMT3* mutations is more prominent in childhood AML cases (Ho, Alonzo et al. 2010, Ho, Kutny et al. 2011, Meshinchi, Alonzo et al. 2006). Furthermore, pediatric AML patients are typically able to tolerate more intensive therapy, which affects the overall outcome compared to adult AML patients.

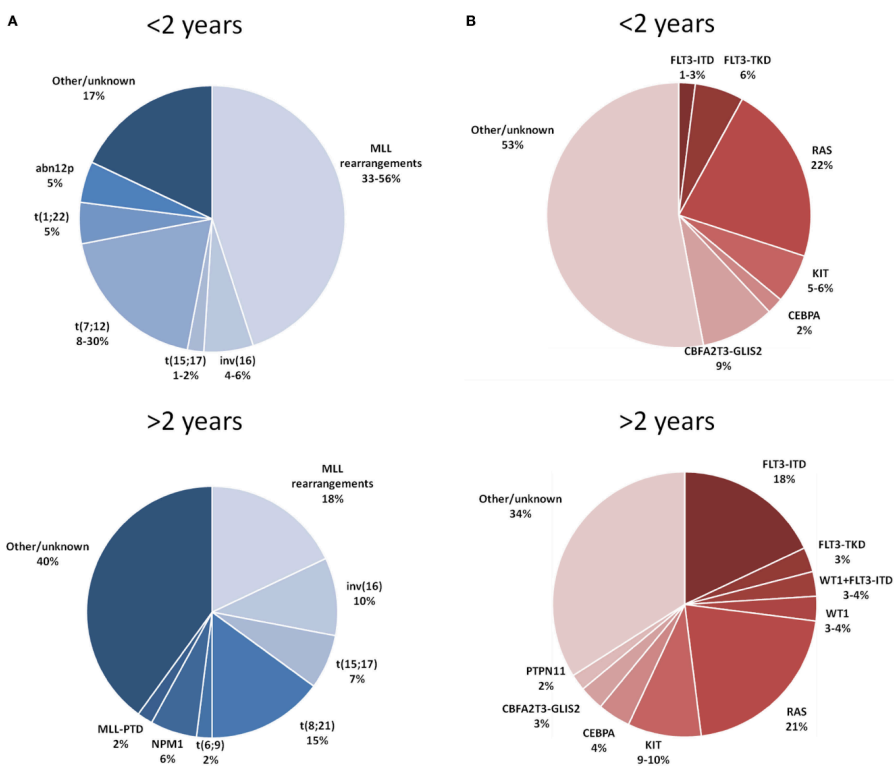

Figure 9. Frequency the main cytogenetic (A) and molecular (B) abnormalities identified in childhood AML younger and older that 2 years old. Figure reprinted with permission from the publisher (Masetti, Vendemini et al. 2015).

2 AIM OF THE THESIS

The overall aim of the thesis was to identify and characterize novel effectors and pathways important in maintenance of AML and in normal hematopoiesis.

The specific aims were:

Study I:

Identify and characterize novel target genes that are specifically required for the maintenance of AML but not normal hematopoietic cells.

Study II:

Identify and characterize novel transcriptional regulators important in the maintenance and disease progression of AML.

Study III:

To investigate the role of the epigenetic enzyme *EHMT1* in AML.

Study IV:

To investigate if targeting SAMHD1 represents an attractive therapeutic approach to potentiate the effects of ara-C treatment in hematological malignancies.

Study V:

To determine the role of the epigenetic factor *NAP1L3* in HSCs and hematopoiesis.

3 METHODOLOGICAL APPROACHES

3.1 LENTIVIRAL TRANSFECTION AND TRANSDUCTION

For lentivirus production, The adherent 293FT cells were co-transfected with the vectors, pAX8 and pCMV-VSVG, using the calcium phosphate transfection method (Kingston, Chen et al. 2003). Briefly, the 293FT cells were split in 10 cm dishes on the day before transfection and the cells fed with 9 ml DMEM media containing 10% FBS 4 hours before transfection. For the transfection 10 µg vector DNA, 8 µg pAX8 and 4 µg pCMV-VSVG were mixed with 64 µl of 2 M $CaCl_2$ and water was added up to 500 µl. 500 µl HEPES was added into a 5 ml FACS tube and the DNA/$CaCl_2$ solution added dropwise with a pipette whilst simultaneously aerating the mixture by constantly bubbling with a mechanical pipettor attached to a 1 ml pipette and then the mixed solution was immediately vortexed for 5 seconds. The precipitate was incubated for 10 minutes at room temperature and 1 ml of the mixed solution was added to a 10 cm plate, covering almost everywhere and incubate for 6 hours or overnight. The medium was replaced with 6 ml of medium suitable for the target cells for transduction, and the transfected plates were incubated at 32℃ to produce virus. Virus supernatant was harvested 24 h and 48 h post transfection and was concentrated by centrifugation at 6000 x g for 16 h at 4℃.

The AML cells and normal hematopoietic cells were transduced with the virus supernatant by spinoculation, i.e. by centrifugation of a mixture of cells and virus for 2 h at 1000 x g in 6 or 12 well non-tissue treated plates. Transduced cells were selected by adding puromycin at the final concentration of 2 µg/ml for 48 h.

3.2 LARGE SCALE SHRNA SCREEN

RNA interference (RNAi) was first discovered in *Caenorhabditis elegans* as double stranded RNA-mediated gene silencing. Double stranded RNA (dsRNA) is a substrate for Dicer (RNAse III enzyme) that produces 20-25 bp dsRNA, which is recognized by the RNA-induced silencing complex (RISC). RISC destroys the “passenger strand” and keeps the “guiding strand”. The RISC complex and guiding strand bind to target mRNA and digest it, resulting in post-transcriptional gene silencing (Gao, Yang et al. 2014).

The major challenge in molecular biology is to find out the functional role of genes in specific strains of cells. RNAi technology (Fire, Xu et al. 1998) is a tool to silence target genes and investigate the role of the gene. The advantage of RNAi is the ability to investigate the role

of a gene based on the phenotype-associated loss of function. Therefore, RNAi has become the appropriate method for functional genomics, signal transduction, and drug target discovery assays. In the cancer field, using RNAi technology helps to identify oncogenes which are essential for cancer cell survival and to find target genes for cancer treatment. Also, it is possible to pool individual RNAi and make libraries for genome-wide or high-throughput screens (HTS) to quickly and simultaneously screen multiple genes. Firstly, the readout of RNAi HTS should be well-defined such as proliferation, apoptosis or cell morphology. Similarly, a proper and easy phenotypic assay should be defined based on the readout.

We used two libraries: library module 1 including 27,500 shRNAs (short hairpin RNAs) against 5,000 signaling pathway genes for study II and library module 2 including 27,500 shRNA targeting disease-associated genes in study I. Each individual shRNA contained a unique barcode.

In studies I and II, we used lentiviral vector-based shRNA screens. To ensure we have enough cells per individual shRNA (>1000 cells/shRNA), we cultured approximately $3x10^8$ of several AML cell lines (THP-1, NOMO-1, and the mouse MLL-AF9 AML) with a concentration of 250,000 cells/ml which were transduced with pooled lentiviral shRNA libraries. To minimize the number of cells with multiple viral integrations, we kept the transduction efficiency at less than 25%, as determined by flow cytometry to measure RFP^+ cells. After 48 h of transduction, we added puromycin (2μg/ml) to select transduced cells, after which, half of these were collected for an initial time point (T0) where the selection control cells were completely dead. The rest of the cells were harvested after 10 cell divisions (T10) in culture. Genomic DNA was extracted from T0 and T10 cells by QIAgene DNA extraction kit according to the manufacturer's instructions, and the barcodes were amplified from the extracted genomic DNA using nested PCR according to Cellecta's protocols. PCR-amplified barcodes were purified using the QIAgene PCR purification kit and used for NGS (HiSeq 2000, Illumina). Barcodes were deciphered using Cellecta's software to detect the number of reads per barcode, which represented the frequency of individual shRNAs targeting specific genes at T0 and T10. After normalization of reads between samples, the ratios of individual barcodes were calculated by dividing T10/T0.

There are three possibilities for each shRNA according the ratio number. If the ratio of T10/T0 is >1, it means that the target gene was a growth suppressor and by knocking down the gene the cells containing the shRNA proliferate more. The second scenario is that T10/T0 is equal to 1, meaning that the target gene did not influence cell growth and finally, the third possibility is that ratio of T10/T0 <1, meaning that the target gene has an important role in

cell growth. We selected the shRNA targeting genes from the third scenario with at least 5-fold reduction (T10/T0 <0.2) in AML cell growth for further validations.

3.3 CRISPR/CAS 9 GENOME EDITING

The clustered regularly interspaced short palindrome repeats (CRISPR)/Cas9 technology is a new gene editing system. CRISPR/Cas9 acts as an adaptive immune system in bacteria for cleaving foreign genetic elements such as viruses and plasmids via RNA-guided nucleases (Deveau, Garneau et al. 2010). Cas9 (a non-specific CRISPR-associated endonuclease 9) is guided by small RNA "gRNAs" to the target DNA sequences. CRISPR systems have been divided into two major classes based on their components and mechanisms of action. Class 1 including type I, III and IV involve several effector proteins but class 2 (type II, V and VI) only requires one RNA-guided endonuclease (Wang, La Russa et al. 2016). Cas9 is guided to target DNA by two hybridized RNAs: crRNAs, that identifies target DNA through a 20 base pair (bp) recognition sequence and tracrRNAs, which hybridizes with the crRNAs. To simplify the system for genome editing, crRNA and tracrRNA, can be engineered as a single chimeric guide RNA (sgRNA) (Jinek, Chylinski et al. 2012). The most widely used Cas9-based genome editing system is a single protein Cas9 with a single RNA sgRNA (Cas9-sgRNA) which binds to 20 bp target DNA adjacent to a protospacer adjacent motif (PAM) sequence (Figure 10).

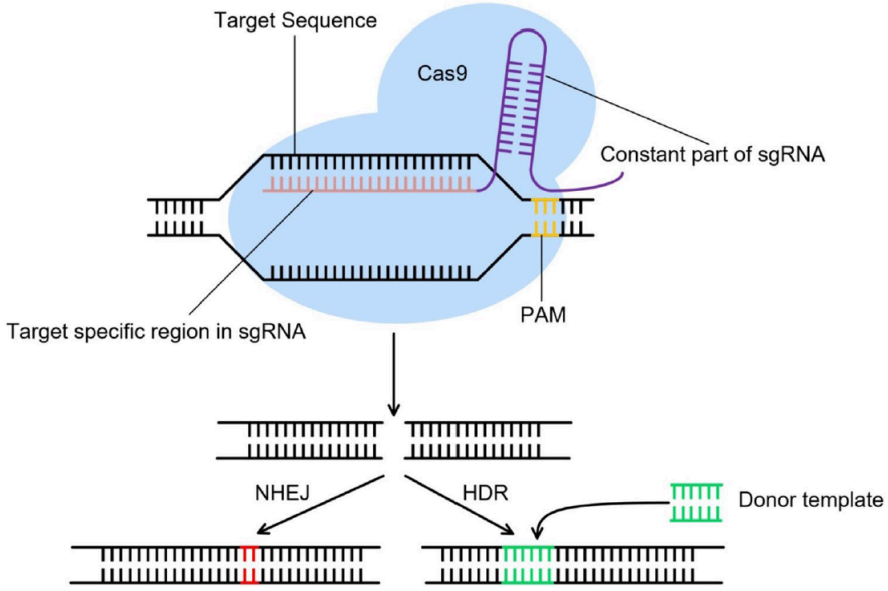

Figure 10. Schematic of the RNA-guided Cas9 nuclease. protospacer adjacent motif (PAM), non-homologous end joining (NHEJ), homology-directed repair (HDR). Figure reprinted with permission from the publisher (Cui, Xu et al. 2018).

CRISPR/Cas9 tools edit the genome via two mechanisms: non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Figure 9). In the absence of a repair template, the Cas9 enzyme creates DNA double strand breaks (DSBs) and re-ligates through the error-prone NHEJ process, often producing an insertion/deletion or single nucleotide mutation, which result in frameshifts and eventually gene knockout. HDR is another DNA repair pathway which uses a repair template which can be used to induce precise and defined gene modification at a target site.

Lastly, since CRISPR/Cas9 is flexible and robust technique, it is becoming attractive tool to use not only for genome editing but for various genome modification applications including gene regulation (silencing or activation), epigenome editing, chromatin imaging, chromatin topology, RNA targeting (Figure 11).

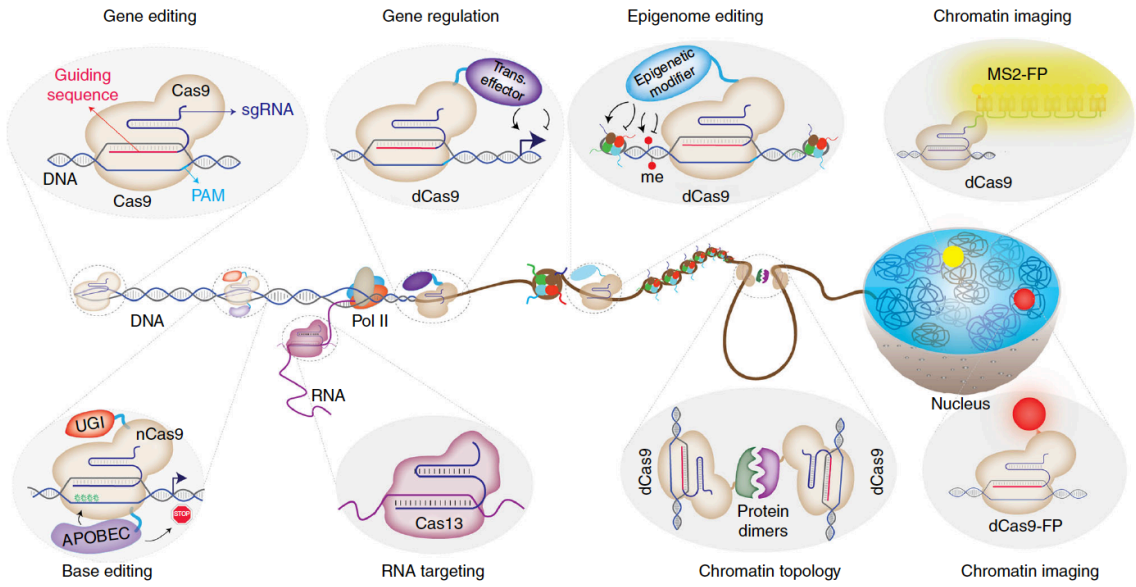

Figure 11. Main applications of CRISPR/Cas9 technology. Figure reprinted with permission from the publisher (Adli 2018).

For CRISPR/Cas9-mediated knockout studies, gRNAs were designed from Optimized CRISPR Design - MIT (http://crispr.mit.edu) provided by the Zhang laboratory and were cloned into the vector for inducible expression pRSITEP-U6Tet-(sh)-EF1-TetRep-2A-Puro expressing fluorescent marker (GFP, BFP, or iRFP670).

First, we developed a stable Cas9 expressing AML cell lines by transducing AML cells with a constitutive expression vector for the Cas9-mCherry fusion protein kindly provided by Marco Herold (Aubrey, Kelly et al. 2015). Then, the transduced cells (Cas9-mCherry^{+}) were sorted using a BD Fusion BSL2 flow cytometer and expanded in culture. For the desired knockout studies, the stable AML cell line expressing Cas9-mCherry was transduced with gRNA vectors expressing either GFP, BFP or iRF670. For tracking the cells with Cas9-

sgRNA, flow cytometry analysis was used to detect double positive cells for example mCherry^{+}iRFP670^{+}.

3.4 FLOW CYTOMETRIC ANALYSIS AND SORTING

Flow cytometry is a potent tool to characterize cells based on size, granularity and fluorescence emission of a targeted antibody. The first flow cytometry analysis was developed in 1956 to detect the size of the cells and in 1980 the first clinical application of a fluorescent activated cell sorter (FACS) was used by researchers to detect lymphocyte subsets (Wilkerson 2012).

The basic principle of a flow cytometry instrument is characterization of cells in a fluid stream passing the cells through the beam of a light source (laser). Therefore, the main components of almost all flow cytometers or cell sorters are: fluidics to direct sheath buffer (phosphate-buffered saline) containing cells to the flow chamber (where the laser beams are located) by pressurized lines, optics including excitation and detection, an electronic network to convert light or fluorescent signals coming from the cells into digital signals and a computer to record digital signals (Figure 12A).

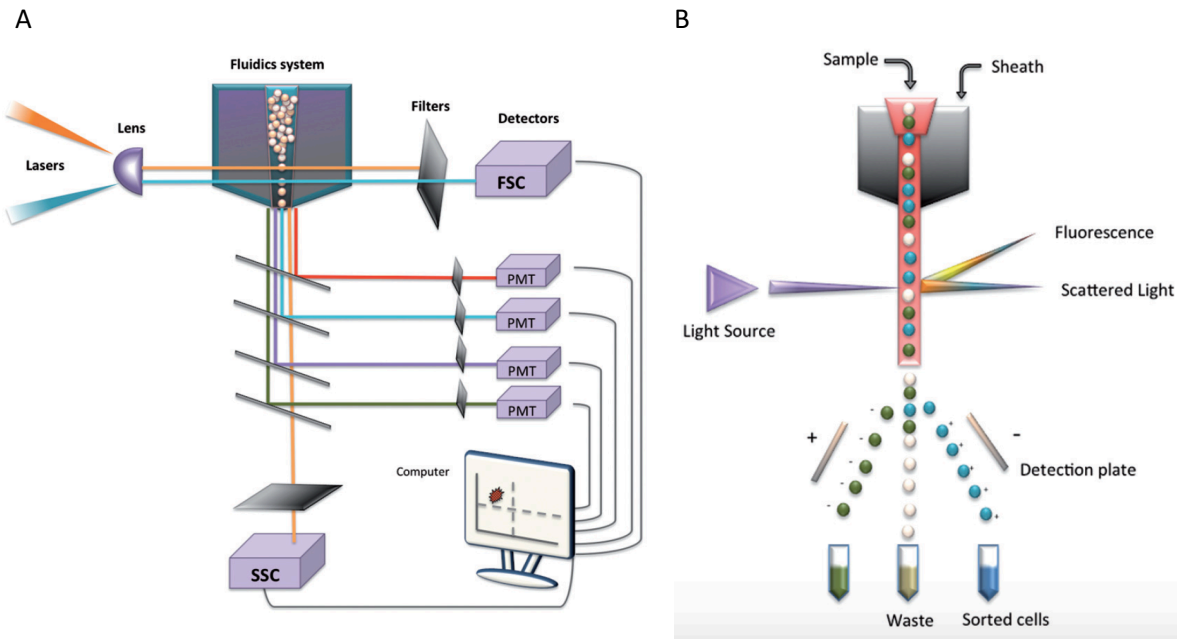

Figure 12. Overview of flow cytometer (A) and sorter (B). Figure reprinted with permission from the publisher (Adan, Alizada et al. 2017).

Flow cytometry is used to characterize cells based on detection of their surface-bound, cytoplasmic and nuclear antigens. Moreover, it is possible to investigate cellular components such as organelles, nuclei, DNA, RNA, cytoplasmic protein, nucleus proteins using flow cytometry.

The principle of cell sorting is an electrostatic deflection of charged droplets containing individual fluorescent cells. Flow sheath buffer containing cells is injected through a vibrating nozzle that break up the stream into droplets surrounding individual cells. Then, the droplets pass through one or more laser beams to characterize the cell population of interest and simultaneously, the tagged droplets are charged by a charging electrode. Then a platinum plate of negative charge deflects positively charged droplets and a positively charged platinum plate deflects negatively charged droplets and uncharged droplets goes into the waste container. Single or multiple parameters can be used for sorting the desired cell population and to collect them for further assays (Figure 12B).

Here flow cytometric analysis was performed with a 4-laser BD LSRFortessa.

To analyze primary childhood and adult AML cells after *ex vivo* culture, the cells were harvested and incubated with anti-mouse (Biolegend) and human (ChromPure Mouse IgG, Jackson ImmmunoResearch) CD16/32 (Fc-block) antibodies for 20 minutes on ice to block unspecific receptors. Then the cells were stained for 20 minutes with anti-human CD45 to distinguish human AML cells from mouse stromal feeder cells, lineage antibodies (CD20, CD4, CD8, CD2, CD56, CD235b, CD3 and CD19) to exclude mature cells and CD34, CD38 to identify LICs. Dead cells were excluded using the Near-IR Live/Dead marker (Invitrogen). To count the absolute number of AML cells in each well from 96-well plate format, we used a high-throughput automated plate reader (BD LSRFortessa).

To detect cells with a specific fluorescent marker mixed with control cells from cell growth competition assays, cells were harvested and washed with cold PBS and thereafter stained with Near-IR Live/Dead marker in a 96-well plate in 80 µl of PBS and 2% FBS. A high-throughput automated plate reader was used to detect the absolute number of live cells.

For *in vivo* studies, and to determine the level of engrafted human AML cells in transplanted NSG-SGM3 mice, the BM cells were isolated by crushing the tibia and femur of recipient mice. The isolated BM cells were incubated in mouse and human FC blocking antibodies for 10 minutes on ice, then stained with human anti-CD45 for 20 minutes. Near-IR Live/Dead marker was used to detect live cells. To analyse the different types of mature human cell populations in NSG-SGM3 transplanted mice, the BM cells were isolated and stained with CD11b, CD14, CD56, CD19, CD33, CD16, CD3, HLA-DR and CD45.

To determine engraftment of mouse CD45.1$^+$ cells in recipient CD45.2$^+$ mice, the BM cells were isolated from tibia and femurs of recipient mice. The isolated cells were then incubated 10 minutes with mouse FC blocking antibody (anti-CD16/CD32), then 20 minutes with mouse antibodies for CD45.1 and CD45.2 to separate donor and recipient cells. To detect percentage of engrafted LSK cells, we added additional anti-mouse lineage markers (CD11b, Gr-1, CD3, CD19 NK1.1 and TER119) together with CD117 (cKit) and Sca-1. Propidium iodide (PI) (Invitrogen) was used to exclude dead cells after washing cells.

All flow cytometry data analysis was done using FlowJo Version 9.3.3 software (TreeStar).

3.5 CELL GROWTH ASSAYS OF AML PATIENT SAMPLES

Long term culturing of the AML samples was carried out as previously reported (Griessinger, Anjos-Afonso et al. 2014). Briefly, mesenchymal MS-5 cells (DSMZ) were irradiated with 80 Gy, washed, and resuspended at a density of 10,000 cells/100µl of Myelocult media H5100 (StemCell Technologies Inc.). To seed feeder cells, 100 µl of cell suspension were plated in a collagen I Cellware 96-well plate (Corning), in triplicates for each condition. 2-3 days after plating feeder cells, 10,000-20,000 AML patient samples were then suspended in Myelocult media supplemented with rhIL-6, rhIL-3, rhFl3/Flk-2 ligand, rhTPO, and rhSCF and rhG-CSF (Stemcell technology) at a concentration of 20 ng/ml, which were added to each well containing a MS-5 monolayer. The plates were kept in a box with water to increase the humidity and maintained under normoxic conditions for 3-5 weeks. The effects on cell growth (bulk or LICs) were determined by flow cytometric analysis.

3.6 ISOLATION AND CULTURE OF PRIMARY NORMAL CELLS

To isolate cKit$^+$ or LSK cells, lineage mature blood cells were depleted from BM cells of the femur and tibias of C57BL/6 mice by staining cells with purified antibodies against Ter119, B220, Gr1, CD3, NK1.1, and CD11b (Biolegend) and Dynabeads (Invitrogen). Fluorochrome-conjugated anti-CD117 (cKit) and anti-Sca-1 antibodies were used to sort cKit$^+$ or LSK cells with a FACS Aria III (BD). The dead cells were excluded by PI (Invitrogen). Normal cKit$^+$/LSK cells were cultured in SFEMII media (Stemcell technology Inc.) including; rhFlt3/Flk-2 ligand (Stemcell technology Inc.), rhTPO (Stemcell technology Inc.), rhIL-6 (R&D system), rmIL-3 (R&D systems) and rmSCF (R&D systems) at a concentration of 20 ng/ml.

For enrichment of normal human $CD34^+$cells, Lymphoprep solution (Invitrogen) was used for the isolation of the mononuclear cell fraction from the umbilical cord blood cells (UCBs) and the $CD34^+$ cells were enriched using a CD34 magnetic activating cell sorting microbead kit (Miltenyi Biotec). Enriched $CD34^+$ cells were cultured in SFEMII media supplemented with rhIL-6, rhIL-3 (R&D systems), rhFl3/Flk-2 ligand, rhTPO and rhSCF all in a final concentration of 20 ng/ml (R&D systems) for 14 days.

3.7 COLONY FORMING UNIT ASSAY

The colony forming unit (CFU) assay is an *in vitro* assay used to investigate proliferation and differentiation patterns of HSCs and progenitors by assessing their capacity to form colonies in a semi-solid medium. The morphology and number of the colonies originating from a certain number of input cells provides information about the proliferation and differentiation ability of the input cells. Moreover, serial plating of each colony illustrates preliminary information about self-renewal capacity of input HSCs.

To investigate the role of a target gene in proliferation and differentiation of HSPCs, knockdown and control groups of normal mouse ($cKit^+$/LSK) or human BM (Lin^- $CD34^+CD38^-$) were resuspended in the MethoCult semi-solid media (Stem Cell Technologies) in concentrations of 150-300 cells/ml (M3434) for mouse and 200-400 cells/ml (M4435) for human. After vortexing the mixture vigorously, 1 ml of the cell mixture was seeded in a $1cm^2$ dish with a 16g gauge blunt-end needle. The colonies were counted and characterized according their morphology using an inverted microscope at 10–12 days (mouse) or 12–14 days (human) after plating. A cluster of more than 50 cells was defined as one colony.

3.8 AML MOUSE MODELS AND TRANSPLANTATION STUDIES

For the *in vivo* part of studies I, II, III and V, all the mice were kept in a specific pathogen-free animal facility at Karolinska Institutet, Huddinge, Sweden. The C57BL/6 wild type mice and the NOD Scid IL2Rgnull-3-SCF/GM/IL3 (NSG-SGM3) mice were purchased from The Jackson Laboratory. All the transplanted mice were monitored daily for symptoms of leukemogenesis and disease progression was investigated using complete blood tests.

The mouse *MLL-AF9* AML cells were generated according to a previous study (Somervaille and Cleary 2006). $cKit^+$ cells were sorted from the BM of $CD45.1^+$ C57BL/6 mice and they were transduced with a retroviral vector expressing the MLL-AF9 fusion oncogene. The

transduced cells were kept in *in vitro* culture and were serially re-plated in semi-solid methylcellulose medium containing 20 ng/ml rmSCF, 10 ng/ml rhIL-6, 10 ng/ml rmGM-CSF, 10 ng/ml rmIL-3 and G418 (0.75 mg/ml) to enrich immortalized cells. Single immortalized clones were expanded and transplanted via the tail vein into sub-lethally irradiated (600 cGy) CD45.2$^+$ C57BL/6 wild type mice. Once transplanted mice showed symptoms of leukemogenesis and disease progression, MLL-AF9 AML cells were harvested from the BM of euthanized primary AML mice, sorted by flow cytometry and expanded in RPMI medium in the presence of IL3 (10 ng/ml) and 10% FBS. The sorted mouse AML cells were used for further *in vitro* or *in vivo* experiments.

For the Kaplan-Meier survival analysis of the AML mouse model, 250,000 mouse *MLL-AF9* AML cells after transduction and selection with puromycin (2 μg/ml) were intravenously transplanted into wild type CD45.2 C57BL/6 mice aged 6-8 weeks. After AML onset, the mice were euthanized.

For *in vivo* experiments with primary human cells including normal hematopoietic cells and AML patient samples, we used sub-lethally irradiated (220 cGy) humanized NSG-SGM3 mice aged 6-10 weeks. The primary cells (normal cells: 25,000-50,000 and AML patient: 100,000-500,000) samples were transplanted via intra-femoral injection. For detection of engrafted cells, the recipient mice were euthanized and the BM cells were collected and analyzed by flow cytometry.

3.9 RNA SEQUENCING

RNA sequencing is a powerful technology to investigate the RNA expression profile of cells. Briefly, extracted RNA, either total or poly-A$^+$ mRNA is converted to cDNA fragments and adaptors are attached in one or two ends of the fragments. The library fragments can be sequenced with high throughput from one end (single-end sequencing) or both ends (pair-end sequencing). The length of the sequenced reads is around 30-400 bp depending on the sequencing technology used. To analyze the reads after sequencing, they are either aligned to a reference genome or reference transcript, or they can be assembled *de novo* without the genome sequence. Then mapped data are normalized and bioinformatic analyses such as expression level, expression of isoforms and whole transcriptome variation and statistical analyses can be performed.

For RNA sequencing in studies I, II, III, and V, total RNA was extracted from cells using the RNeasy Micro Kit (Qiagen) 72 hours after transduction of the cells by sorting or selection

with puromycin. TotalScriptTM RNA-seq kit (Epicentre, Madison, WI) was used to prepare strand specific pair-end RNA libraries according to the manufacturer's instructions. Libraries were pair-end sequenced using the Illumina platform HiSeq2000 or Nextseq500. RNA-seq reads were mapped to the Ensembl Homo sapiens GRCh38 reference genome using the STAR aligner. Gene assignment was performed using feature Counts. Normalization and the sample group comparison was performed using DESeq2.

4 RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Study I

In this study, we aimed to identify novel target genes that are selective and essential for maintenance of AML. For this purpose, we performed shRNA based screens of the human AML cell lines NOMO-1 and a mouse AML cell line. All cell lines were characterized by MLL-AF9 translocation. To filter out targets with a strong effect on normal hematopoietic cells we used non-transformed mouse Factor-Dependent Continuous Paterson Laboratories (FDCP mix) cells as control cells in the screens (Spooncer, Heyworth et al. 1986). The AML cells were transduced with lentiviral virus from an RNAi library consisting 27,000 shRNAs targeting 5,400 putative disease-associated and drug targets (Cellecta Inc.). The transduced cells were harvested at the initial time point (T0) and after ten cells divisions (T10) to sequence the PCR-amplified unique barcodes for individual shRNAs. By comparing the ratio of each individual barcode at two time points, we identified 1,082 target genes with at least a five-fold higher inhibitory effect on mouse AML cell growth compared to FDCP-mix cells. Among the 1,082 targets, 34 were common between THP-1 and NOMO-1 cell lines as shown by at least a five-fold effect in the screens.

Among 34 target genes, multiple shRNAs were scored against chromodomain helicase DNA-binding protein 4 (CHD4) which had a strong growth inhibitory effect on AML cell lines. To validate whether CHD4 is required for AML cell growth, we performed an *in vitro* competition assay by mixing shRNA expressing RFP and targeting *Chd4* with control shRNA tagged with a GFP fluorescent marker. Knockdown of *Chd4* with two independent shRNAs caused a strong blockade of leukemic cells in THP-1, NOMO-1 and mouse AML cells. We then tested the effect of *CHD4* knockdown in non-MLL AML cells lines by using HL-60, K562 and NB-4 AML cell lines. The result from this *in vitro* growth assay revealed that *CHD4* is also crucial for growth of non-MLL AML cells. We used CRISPR/Cas9 technology to target *CHD4* on two human AML cell lines (THP-1, MV4-11) to avoid possible off-target effects commonly caused by shRNA, and confirmed that *CHD4* expression is crucial for propagation of AML cells.

To investigate whether CHD4 plays role in AML progression, we transplanted MLL-AF9 AML cells into recipient congenic mice. The mice which received *Chd4* knockdown AML cells survived significantly longer than the control group transplanted with AML cells

carrying Sc control shRNA. To check whether *CHD4* is important for normal hematopoietic cells, we first tested the effect of knockdown on normal mouse HSPCs in a growth assay format. The flow cytometric analysis demonstrated a minor difference between cells transduced with *Chd4* shRNA and control cells. Moreover, a similar result was seen when testing *CHD4* shRNA in human CD34 positive UCBs. Together, these results indicate that *CHD4* plays an important role in the growth of leukemic cells *in vitro* but not in normal primary hematopoietic cells.

Next, we tested knockdown of *CHD4* shRNA in primary childhood patient samples *ex vivo* by culturing transduced patient samples either with control shRNA or *CHD4* shRNA on MS5 stromal feeder cells. Flow cytometry analysis demonstrated that the expansion of cells transduced with *CHD4* shRNA was significantly inhibited compared to control cells, whilst the absolute number of LICs were decreased in *CHD4* knockdown cells. These results suggest that *CHD4* inhibition prevents expansion of bulk leukemic cells as well as LICs. Interestingly, using a humanized NSG-SGM3 mouse model to transplant childhood patient samples transduced with either *CHD4* shRNA or Sc control showed that primary childhood AML cells transduced with *CHD4* shRNA had significantly lower levels of leukemic engraftment in recipient mice compared to the Sc control group.

Cell cycle assay analysis of MLL rearranged AML (THP-1 and MV4-11) and non-MLL AML (AML-193 and Kasumi-1) human cell lines revealed that inhibition of *CHD4* caused an accumulation of cells in G0 phase of cell cycle. Additionally, *CHD4* inhibition reduced the number of cells in G1 phase but did not significantly affect the number of cells in S and G2/M phases compared to control cells. Contrastingly, a minor effect on apoptotic cells was observed using *CHD4* shRNA.

Next, to determine the RNA expression pattern induced by *CHD4* knockdown, we performed RNA sequencing analysis of THP-1 AML cells transduced with *CHD4* shRNA. Gene set enrichment analysis (GSEA) of RNA-seq data showed that the genes most significantly correlated with *MYC* and *E2F* targets. Moreover, knocking down of *CHD4* induced downregulation of *MYC* and genes involved in G1/S cell cycle such as D1, D2, E1, E2F1 and E2F2.

In this study, we found that the shRNAs targeting *CHD4* identified from our screens targeted AML cells without a dramatic effect on normal hematopoietic cells, making *CHD4* a potential therapeutic option for AML. We showed that *CHD4* knockdown reduced the frequency of bulk AML cells as well as LICs in primary childhood AML samples. Additionally, we determined that *CHD4* is essential for AML progression and development

by testing the effect of knockdown in a congenic AML mouse model and transplanting patient samples into NSG-SGM3 mice. These data revealed an important role for *CHD4* in childhood AML.

4.1.2 Study II

In this study, our goal was to identify novel TFs essential for AML cell expansion. We screened two human cell lines (THP-1 and NOMO-1) and mouse AML cells with a lentiviral shRNA library targeting around 5,000 genes within signaling pathways (Cellecta Inc.). The transduced cells were harvested at two time points; initial and after 10 cell divisions. Each individual shRNA vector contained a unique barcode which was amplified from genomic DNA and tracked by NGS. We found 648 target genes which caused at least a five-fold reduction in AML cell growth overlapping between THP-1, NOMO-1 and mouse AML cells. Among the target genes, 38 of them were TFs and 24 of the 38 were known to be important in AML such as *MEIS1*, *HEMS1* and *MYC*. To avoid strong effects on normal cells, we prioritized expression levels of TFs in myeloid leukemia cells and normal hematopoietic cells as listed in the BloodSpot data base. *GTF2IRD1* is one such TF that was highly expressed in leukemic cells.

To validate *GTF2IRD1*, we first tested mouse AML cells by performing growth assays using AML cells transduced with shRNAs targeting *Gtf2ird1*, mixed with cells transduced with Sc control shRNA. FACS analysis showed *Gtf2ird1* knockdown significantly impaired AML growth. We found a similar result in five human AML cell lines; THP-1, NOMO-1, HL-60, K-562, and NB-4. Then we investigated the importance of *Gtf2ird1* in AML development *in vivo* using a congenic AML mouse model. The mice transplanted with *Gtf2ird1* knockdown AML cells survived longer compared to the recipient mice transplanted with AML cells transduced with Sc control shRNA.

Our next question was if GTF2IRD1 blocks the growth of normal hematopoietic cells. To answer this question, we first performed *in vitro* growth assays on mouse HSPCs. FACS data showed no significant difference between cells transduced with *Gtf2ird1* shRNA and control cells. Furthermore, the CFU assay confirmed *Gtf2ird1* is not essential for expansion and differentiation of mouse HSPCs *in vitro*. Transplantation of depleted *Gtf2ird1* HSPCs into lethally irradiated mice and monitoring the frequency of engraftment demonstrated that knockdown of *Gtf2ird1* did not strongly affect the reconstitution of HSPCs cells *in vivo*.

To explore the effect of *GTF2IRD1* inhibition on survival and expansion in normal human hematopoietic cells, we performed a growth assay on $CD34^+$ cells enriched from UCBs

transduced with *GTF2IRD1* shRNA or Sc shRNA. Similar to the results observed with mouse HPSCs, we again saw that *GTF2IRD1* is not crucial for expansion of human hematopoietic cells. Long-term culturing of transduced $CD34^+$ UCBs cells also revealed *GTF2IRD1* inhibition did not influence normal cell growth. Transplantation of $CD34^+$ UCBs into NSG-SGM3 mice and FACS analysis of engrafted cells after 8 weeks demonstrated no significant differences in engraftment between the mice receiving cells with *GTF2IRD1* knockdown compared to the Sc control group. Altogether, data from mouse and human HSPCs suggest that the *GTF2IRD1* TF has a selective role in the expansion and development of AML but is not important in normal hematopoiesis.

Additionally, we knocked-out *GTF2IRD1* in THP-1 and MV4-11 cells using CRISPR/Cas9 technology and performed an *in vitro* competition assay. By comparing cells with gRNA targeting *GTF2IRD1* to cells transduced with control gRNA, we observed a strong reduction in *GTF2IRD1* knockout cells which confirmed our result as previously seen in AML cells using shRNA.

To investigate the role of *GTF2IRD1* in primary childhood and adult AML patients, we took advantage of *ex vivo* culturing of AML samples on stromal feeder cells. Transduction of patient samples with *GTF2IRD1* shRNA caused a significant reduction of bulk AML ($CD45^+$) cells as well as LICs ($CD45^+Lin^-CD34^+Cd38^-$). Additionally, intrafemoral transplantation of transduced cells into NSG-SGM3 humanized mouse cells showed *GTF2IRD1* knockdown decreased the percentage of engrafted adult and childhood AML samples. Altogether, these data confirm that expression of *GTF2IRD1* is crucial for the survival and maintenance of adult and childhood primary AML cells.

Cell cycle and apoptosis analysis of mouse AML cells revealed a significant increase in the number of cells in the G0 cell cycle phase and reduction in G1, S and G2M phases caused by knocking down of *Gtf2ird1* compared to control cells. In contrast, the percentage of apoptotic cells at the same time point was low, around 2-3%, suggesting that *Gtf2ird1* knockdown induces an accumulation of cells in the G0 phase of the cell cycle rather than inducing apoptosis.

To find out the role of *GTF2IRD1* in gene regulation, we used a heterologous GAL4 reporter system (Hansen, Bracken et al. 2008). In this system, the luciferase reporter gene is regulated by a thymidine kinase promoter with five GAL4 binding sites, therefore suppressor and activation function of a TF fused with GAL4 can be measured by luciferin expression level. Tetracycline induction of *GTF2IRD1-GAL4* expression suppressed luciferase expression levels, suggesting that *GTF2IRD1* TF acts as a transcriptional suppressor in AML cells.

RNA sequencing of mouse AML cells with *Gtf2ird1* knockdown compared to control cells revealed that *Gtf2ird1* knockdown mRNA profiles were enriched in various cellular pathways, including myeloid leucocytes activation, lipid biosynthesis and cell-cell adhesion. Furthermore, GSEA showed knockdown of *Gtf2ird1* negatively correlated with *MYC* targets data sets (FDR q value=0.0 and NES = -1.7) and KRAS signaling (FDR q value = 0.0, NES -1.6).

4.1.3 Study III

Here we focused on the role of euchromatin histone methyltransferases 1 (EHMT1) in AML. EHMT1 and EHMT2 are enzyme homologs which specifically methylate the lysine 9 (K9) residue in histone 3 (H3), acting as transcription repressors. Several studies have shown the function of EHMT2 in AML (Lehnertz, Pabst et al. 2014), but the role of EHMT1 in AML is not currently clear.

To first investigate the importance of *Ehmt1* in AML, we knock downed *Ehmt1* using shRNA in mouse MLL-AF9 AML cells and analyzed the growth of AML cells in competition with control cells. Monitoring cells via flow cytometry analysis for two weeks showed that knockdown of *Ehmt1* significantly reduced the frequency of AML cells *in vitro*. To further analyze the role of *Ehmt1* in mouse AML cells, we transplanted AML cells into lethally irradiated recipient mice with and without knockdown of *Ehmt1*. Interestingly, transplanted cells containing one of the independent shRNAs targeting *Ehmt1* prolong the survival of mice in comparison to mice transplanted with control cells, which died 18-21 days after transplantation. These data suggest that expression of *Ehmt1* is necessary for the growth and progression of mouse AML cells.

Next, we studied the importance of *EHMT1* in human AML cells lines by transducing them with shRNA against *EHMT1*. Growth assay analysis of five AML cells lines with MLL-AF9 translocation (THP-1 and NOMO-1) and without MLL-AF9 alteration (HL-60, K-562 and NB-4) revealed that the essential role of *EHMT1* in mouse AML cell expansion was conserved in human AML cells as well. Moreover, suppression of *EHMT1* activity with the small inhibitor molecule; BIX-01294 in human KL-60 and THP-1 AML cells showed similar results in line with *EHMT1* shRNA; namely that inhibition of *EHMT1* significantly impairs the growth of AML cells.

To confirm the results observed using shRNA, we used CRISPR/Cas9 to knockout *EHMT1* in human AML cell lines. We transduced THP-1 and MV4-11 cell lines with stable expression of Cas9-mCherry using a gRNA targeting the *EHMT1* gene. Targeting *EHMT1*

with gRNA caused a significant reduction in AML cell growth, confirming our previous result regarding the important role of *EHMT1* in AML cell growth.

To find out whether *EHMT1* is important for normal hematopoietic cell propagation we tested the effect of *EHMT1* knockdown on mouse and human HSPCs. In both cases we observed a reduction in cell growth, but this reduction in cell number was not as strong as seen in AML cells. To further investigate, we performed CFU assays for normal mouse and human HPSCs with and without *EHMT1* knockdown. The results revealed *EHMT1* suppression caused a reduction in colony number. Together, these data suggest that AML cell growth is more dependent on *EHMT1* compared to normal HSPCs.

Next, to demonstrate the role of *EHMT1* in primary AML adult and childhood patient samples, we co-cultured transduced cells with either *EHMT1* or Sc shRNA with MS5 stromal feeder cells (Griessinger, Anjos-Afonso et al. 2014). Flow cytometry analysis to read out the frequency of bulk AML cells ($CD45^+$) and LIC ($Lin^-CD34^+CD38^-$) showed that *EHMT1* knockdown AML cells expanded less compared to control cells. To further investigate the role of *EHMT1* in the expansion of primary AML cells, we transplanted *EHMT1* knockdown cells into a NSG-SGM3 mouse model and monitored the engrafted cells by flow cytometry up to eight weeks post transplantation. This was in comparison to control mice receiving AML cells transduced with Sc shRNA. Engraftment of primary patient samples was significantly lower in the group with EHMT1 knockdown. Therefore, these data showed *EHMT1* also plays an important role in the growth of primary AML samples in *in vitro* and *in vivo* conditions.

EHMT2 is another member of the EHMT family, described as a heterodimer to *EHMT1* (Tachibana, Ueda et al. 2005), with important roles in AML previously reported (Lehnertz, Pabst et al. 2014). To address the question if *EHMT1* and *EHMT2* have an overlapping role in AML, we designed gRNAs against *EHMT1* or *EHMT2* with different fluorescent reporter markers, enabling us to track them in co-culture conditions and to detect cells carrying both gRNAs. Flow cytometry analysis of the competition assay revealed that single knockout of *EHMT1* or *EHMT2* reduced AML growth equally and interestingly, double knockout of *EHMT1* and *EHMT2* did not show an additive effect on AML cell growth. These data suggest that *EHMT1* and *EHMT2* have a cooperative function in the growth of AML cells.

To understand the cellular mechanism of *EHMT1* and *EHMT2* in AML cells, we performed cell cycle analysis on mouse MLL-AF9 AML cells with and without knockdown for either *Ehmt1* or *Ehmt2*. Interestingly, we observed both *Ehmt1* and *Ehmt2* knockdown caused a significant increase in the proportion of cells in the G0 phase of the cell cycle and

simultaneous reduction of cells in S and G2M phases. However, we did not observe a significant difference in the number of apoptotic cells, suggesting both *EHMT1* and *EHMT2* inhibition induce cell cycle arrest in AML cells in G0.

To uncover the molecular mechanism of *Ehmt1* in the regulation of AML cell growth, we RNA sequenced mouse AML cells with *Ehmt1* knockdown. Additionally, to detect similarities and differences in the RNA expression profiles between *Ehmt1* and *Ehmt2*, we performed RNA-seq analysis of *Ehmt2* knockdown AML cells. The mRNA profile of *Ehmt1* showed a significant correlation to the *Ehmt2* mRNA profile and since both enzymes are transcriptional suppressors, they unsurprisingly showed a significant overlap in the upregulated genes. Moreover, GO-term analysis of upregulated genes revealed common biological processes associated to cytokine signaling, inflammatory response and cell differentiation for *Ehmt1* and *Ehmt2*. These RNA-seq data suggest although Ehmt1 and Ehmt2 share several biological processes, they may still have further independent roles as well.

4.1.4 Study IV

In this study, we contributed to Nikolas Herold's study to investigate the effect of SAMHD1 inhibitor Vpx, in the treatment of patient AML samples with the cytostatic deoxycytidine analog cytarabine (ara-C). ara-C is the most common drug for AML treatment and resistance to ara-C is the main cause of CR failure and relapse.

SAMHD1 was identified as a risk factor in cohorts of both childhood and adult AML patients who received ara-C treatment. Using simian immunodeficiency virus (SIV) protein Vpx to transiently reduce SAMHD1 expression, AML cells demonstrated a markedly increased sensitivity to ara-C-induced cytotoxicity. Moreover, disruption of *SAMHD1* using CRISPR/Cas9 technology showed a similar effect in increasing sensitivity of AML cells to ara-C.

Xenotransplantation of THP-1 cells with or without expression of *SAMDH1* into athymic nude immunodeficient NMRI nu/nu mice and treatment of the animals with ara-C revealed that SAMHD1^{-} tumors responded to the treatment compared to SAMHD1^{+} tumors.

Next, we treated adult and childhood AML patient samples under *ex vivo* conditions with virus-like particles (VLPs) either with or without Vpx. Flow cytometric results revealed that reduction of SAMHD1 protein levels in patient AML samples increased sensitivity of cells

to ara-C. We also showed that Vpx increased the number of apoptotic cells compared to cells treated with empty VLP.

Thus, a low expression level of SAMHD1 is associated with sensitivity of leukemic cell to ara-C, suggesting that the targeting of SAMHD1 could be used as a potential therapeutic strategy to increase ara-C efficacy in AML patients.

4.1.5 Study V

Epigenetic regulators play an important role in hematopoiesis. In particular, the self-renewal capacity and differentiation abilities of HSCs, acting as the main source of all mature blood cells are regulated with epigenetic regulators. In this study, we investigated the role of *NAP1L3*; nucleosome assembly proteins in self-renewing and differentiation of HSCs *in vitro* and *in vivo* and signaling pathways regulated by *NAP1l3* in hematopoiesis. *NAP1L3* has been reported as one of the 36 transcriptional regulatory genes expressed predominantly in HSCs, suggesting a potential role in HSC regulation (Riddell, Gazit et al. 2014). To detect the expression level of *Nap1l3* in different populations of mouse hematopoietic and progenitor cells, we measured the *Nap1l3* mRNA level in seven FACS sorted populations; HSC ($Lin^-Sca1^+cKit^+CD105^+CD150^+$), multi-potent progenitors (MPP; $LSK^+CD105^+CD150^+$), lymphoid-primed multipotent progenitors (LMPP; LSK^+Flk2^{high+}), common lymphoid progenitors (CLP; $Lin^-IL7Ra^+flk2^+$), pre-granulocyte-macrophage progenitors (pre-GM; $LSK^-CD41^-CD150^-CD105^-$), granulocyte-monocyte progenitors (GMP; $LSK^-CD41^-CD150^-FcgR^+$), and erythrocyte progenitors (pre-CFU E; $LSK^-CD41^-CD105^+$). Consistent with previous studies, qPCR analysis showed that *Nap1l3* was exclusively expressed in the HSC fraction.

To explore the importance of *Nap1l3* in HSPCs proliferation and differentiation, shRNA-based knockdown was used to study loss of function of *Nap1l3*. Knockdown of *Nap1l3* in FACS sorted mouse $Lin^-Sca^+cKit^+$ (LSK) cells caused a significant reduction of total CFUs, as well as mixed myelo-erythroid CFUs (CFU-GEM) and granulocyte/macrophage CFUs (CFU-GM) numbers compared to the LSK cells transduced with negative scramble control shRNA. These data suggest that *Nap1l3* suppression impairs the proliferation and survival of mouse HSPCs *in vitro*.

To avoid potential off-target effects associated with shRNAs, we designed gRNAs to target *Nap1l3* via CRISPR/Cas9 technology. LSK cells were sorted from transgenic mice overexpressing Cas9 nuclease and then transduced with gRNA vectors against *Nap1l3*.

Consistent with shRNA-mediated knockdown, knockout of *Nap1l3* with CRISPR/Cas9 caused a significant reduction in the CFU colony number compare to control cells.

Next, we used exogenous expression vectors to induce expression of *Nap1l3* in mouse HSPCs. Constitutive overexpression of *Nap1l3* caused a significant reduction of the total number of CFUs, CFU-GM and CFU-GEM colonies.

To investigate this further, we transplanted sorted (ckit$^+$) HSPCs transduced with *Nap1l3* shRNA or Sc shRNA into congenic lethally irradiated mice. We then monitored the engrafted cells (CD45.1$^+$) in the BM of recipient mice 2, 5, 8 and 16 weeks post-transplantation. Flow cytometric analysis revealed knockdown of *Nap1l3* caused a significant reduction in engraftment. Moreover, by deep analysis of the BM population, we showed a distinct reduction in LSK cells mediated by *Nap1l3* downregulation. These data suggest that *Nap1l3* plays an important role in the survival and reconstitution of HSC in both the short- and long-term *in vivo*. Furthermore, flow cytometric analysis of mature blood cells showed a decrease in the frequency of myeloid cells (CD11b$^+$), granulocytes (Gr-1$^+$) and, contrastingly, an increase B cells (CD19$^+$) in *Nap1l3* knockdown cells. Altogether, these data show *Nap1l3* may have a role in differentiation regulation of HSCs.

To dig in to the importance role of *NAP1L3* in human HSCs, we transduced enriched HSCs (Lin$^-$CD34$^+$CD38$^-$) from UCB cells with two shRNAs against *NAP1L3* and Sc control. Proliferation analysis of these cells after 48 hours showed that *NAP1L3* knockdown impaired proliferation and reduced the number of mature cells (Lin$^+$) compared to control cells, suggesting that *NAP1L3* is required for proliferation and differentiation of HSC *in vitro*. Culturing UCB HSCs with *NAP1L3* inhibition on stromal feeder cells (SL/SL and M2-10B4) for three weeks showed a significant reduction in CD45$^+$, Lin$^-$CD45$^+$, and UCB HSCs. Also, CFU assays on normal human UCB HSCs demonstrated that knockdown of *NAP1L3* significantly reduced the number of colonies as well as burst-forming unit erythroid cells (CFU-E/BFU-E), macrophages (CFU-M), granulocytes/macrophages (CFU-G/GM), and mixed myelo-erythroid cells (CFU-GEM). To study the effect of *NAP1l3* in HSC self-renewal, we performed a serial plating assay. In doing so, we observed a reduction of colony numbers in the first, second and third plating which suggests *NALP13* may also be important for HSC self-renewal.

To study the cellular mechanisms controlled by *NAP1L3*, we performed cell cycle analysis and apoptosis assays. Cell cycle analysis of UCB HSCs with *NAP1L3* knockdown compared to control cells revealed an accumulation of cells in the G0 phase and a reduction of cells in

G1. Apoptosis assays of cells showed an increased population in early apoptosis. Altogether, knockdown of *NAP1L3* induced cell cycle arrest of HSCs in G0 and triggered apoptosis.

Next, we wanted to see the role of *NAP1L3 in vivo*, so we transplanted $CD34^+$ HSPC UCBs transduced with *NAP1L3* or Sc shRNA into humanized NSG-SGM3 mice. Flow cytometric analysis of BM cells in recipient mice 8 weeks post-transplantation showed that *NAP1L3* knockdown dramatically reduced engraftment percentage. FACS analysis of human mature cells in recipient mice also showed an increase in the percentage of myeloid cells (monocytes, dendritic like cells, plasmacytoid dendritic cells) compared to the control group. Therefore, *NAP1L3* is likely to play an important role during HSC self-renewal and differentiation.

To uncover the mRNA expression profile induced by *NAP1L3* knockdown, we performed RNA sequencing of sorted UCB HSCs transduced with *NAP1L3* compared to cells transduced with control Sc shRNA. GSEA analysis of Biocarta gene set pathways revealed that the mRNA profile of cells with *NAP1L3* knockdown negatively correlated to cell cycle regulation, chromosome function, recombination and replication. We also observed *NAP1L3* knockdown induced upregulation of the *HOXA* cluster in RNA-seq data, which we confirmed by qPCR for all *HOXA* clusters except *HOXA4*. Flow cytometry also revealed that protein levels of HOXA3 and HOXA5 were upregulated in the presence of *NAP1L3* shRNA. In conclusion, these data show that downregulation of *NAP1L3* in UCB HSCs induces gene expression signatures associated with cell cycle progression and *HOXA* gene expression.

4.2 DISCUSSION

To design new drugs to eradicate cancer cells, it is very important to selectively target the cancer cells without eliminating normal cells. In studies I and II, we found that *CHD4* and *GTF2IRD1* play critical roles in leukemic cell growth in various AML cell lines with different mutational backgrounds and in AML development in mouse models. Interestingly, they are not pivotal for the normal function of hematopoietic cells.

In **study I**, we identified the chromatin remodeler *CHD4* from shRNA screens of mouse and human AML cell lines and non-transformed FDCP cells. Our results illustrate a novel and crucial role of *CHD4* in the maintenance and progression of childhood AML by testing the patient samples in an *ex-vivo* co-culture system and a patient-derived xenograft mouse model. However, *CHD4* knockdown did not show a significant effect on normal mouse and human hematopoietic cell growth. Our findings do emphasize a vital role for *CHD4* in the expansion of primary childhood AML and interestingly in the maintenance of LICs. Using immune

phenotyping of surface markers to identify LICs, we found that expression of CHD4 is important for them, which is in line with other cancer types (Chudnovsky, Kim et al. 2014, Nio, Yamashita et al. 2015). Knockdown of *CHD4* revealed an important role of this epigenetic factor in the growth of AML by arresting cells in G0. We showed *CHD4* inhibition causes cell cycle arrest in G0, and our results are supported by other findings recognizing the role of CHD4 in the cell cycle (O'Shaughnessy and Hendrich 2013, Polo, Kaidi et al. 2010). To illustrate how *CHD4* affects the cell cycle, our RNA-seq data revealed a significant downregulation of *MYC* and its target genes under *CHD4* knockdown conditions, which suggest that the *MYC* gene may be a downstream target of *CHD4* which causes cell arrest in G0. To this end, our findings illustrate CHD4 as a potential therapeutic target, which can be used to selectively eliminate childhood AML cells and to particularly diminish LICs.

In **study II**, we found a novel TF, GTF2IRD1, from large scale shRNA screens and we demonstrated that both knockdown and knockout of *GTF2IRD1* selectively blocked mouse and human AML growth. We found a novel role of TF *GTF2IRD1* in maintenance and growth of both childhood and adult AML. Although overexpression of *GTF2IRD1* was reported in AML cells compared to normal hematopoietic cells, our results revealed for the first time the functional role of *GTF2IRD1* in leukemic cells as well as in LICs growth in adult and childhood AML patient samples (Bagger, Sasivarevic et al. 2016). Though more investigations are needed to uncover the molecular mechanism of *GTF2IRD1* in the growth of leukemic cells, our results suggest a selective inhibitory role in AML cell expansion but not in normal hematopoietic cells. Additionally, *GTF2IRD1* knockdown caused an accumulation of AML cells in G0 and downregulation of MYC targets and KRAS signaling. Despite the transcriptional repressor function of *GTF2IRD1*, RNA-seq data from AML cells with *Gtf2ird1* knockdown revealed a strong downregulation of *MYC* targets at the mRNA level, which might correlate with a secondary side effect of blocking cells in G0 upon *Gtf2ird1* knockdown rather than a direct regulatory role of *MYC* target genes. Recently, it has been possible to target TFs in new therapies for cancer cells by modifying their expression or degradation at the mRNA level, by inhibiting protein-protein interactions, or by direct targeting TF and block binding the TF to DNA (Lambert, Jambon et al. 2018). In particular, there are several efforts to develop new strategies to target different TFs in AML including C/EBPα, PU.1, RUNX1, RUNX2, RUNX3, p53, c-MYC, CREB and STAT3 (Takei and Kobayashi 2018). Most importantly, we have identified a novel role of *GTF2IRD1* in AML cell growth and maintenance, suggesting a new TF as a promising therapeutic candidate to selectively target AML cells.

The aim of **study III** was to investigate the role of histone methyltransferase *EHMT1* in AML and to establish a connection to its homolog *EHMT2*. We showed that downregulation of *EHMT1* had a negative effect on maintenance of AML cells in both *in vitro* and *in vivo*. Knockdown of *EHMT1* reduced the number of bulk and LIC cells in primary AML patient samples. Our results suggest that expression of *Ehmt1*, similar to *Ehmt2*, is vital for proliferation of AML cell lines as well as patient-derived AML samples. Simultaneous knockout of *EHMT1* and *EHMT2* in AML cells did not show an additive effect on cell growth compared to single knockout, suggesting that *EHMT1* and *EHMT2* functionally overlap in the regulation of AML growth. Our studies also revealed that both *Ehmt1* and *Ehmt2* knockdown arrests cells in G0 of the cell cycle. Furthermore, our RNA-seq data showed significant overlap in upregulated genes between *Ehmt1* knockdown AML samples and *Ehmt2* knockdown samples. This is consistent with a previous study showing that *Ehmt1* and *Ehmt2* predominantly form a heterodimer complex in the nucleus and are both cooperatively important for H3K9 methylation as an inactivating epigenetic marker (Tachibana, Ueda et al. 2005). Besides that, our data also revealed several upregulated genes and pathways that are not shared between the two groups of knockdown cells, suggesting *Ehmt1* and *Ehmt2* might play independent roles in AML cells in addition to their cooperative role. Since both epigenetic enzymes are associated with transcriptional inactivation, downregulated genes most likely represent secondary effects of *Ehmt1* or *Ehmt2* knockdown. Further experiments need to be done to clarify the functional relationship between the two epigenetic enzymes and promoter regions they bind to together or individually, but it is possible to find homodimer complexes of *Ehmt1* and *Ehmt2* with specific regulatory sites in AML cells. Also, more investigations are clearly needed to identify which of the two H3K9me1/2 methyltransferases shows less pronounced effects on normal hematopoietic cells. Based on the results, studies can be performed to design a specific inhibitor that selectively blocks either EHMT1 or EHMT2 as therapeutic candidate in AML.

In **study IV**, resistance to ara-C and relapse after treatment are the main obstacles to achieve long term CR in AML patients. Therefore, finding a prognostic marker to predict chemotherapy response will be valuable for AML patients. Here, the data suggested that there is a significant correlation between high level expression of *SAMHD1* and low sensitivity to ara-C in AML patient samples, suggesting the correlation between SAMHD1 expression and prediction of ara-C treatment response. The increased sensitivity to ara-C in the absence of SAMHD1 could be because of elevated levels of intracellular ara-CTP, which cause increased incorporation of ara-C into DNA. However, there is no significant difference in SAMHD1 levels between AML patients with CR and patients who did not achieve CR. One

explanation could be clonal selection of ara-C resistant sub-clones with higher levels of SAMHD1 after therapy that might induce relapse. Moreover, the probable tumor suppressor function of SAMHD1 might increase the survival advantage of sub-clones with low SAMHD1 expression level over time (Clifford, Louis et al. 2014). Importantly, our results suggest that expression of SAMHD1 may be a limiting factor for ara-C therapies and evaluation of the level of SAMHD1 could be helpful to avoid toxicity related to increasing doses of ara-C. Further efforts are needed to explore strategies to inhibit SAMHD1 in combination with ara-C treatment, but these data suggest that leukemic cells can be sensitized to ara-C with inhibition of SAMHD1.

In **study V**, our investigations recognized a novel role of the histone chaperone *NAP1L3* in HSCs. Loss of function of the highly expressed *NAP1L3* in HSPCs significantly impaired survival, proliferation, reconstitution and differentiation of HSCs *in vitro* and in a transplanted mouse model. These data suggest the transcriptional regulatory role of *NAP1L3* on genes that are associated with cell cycle and differentiation processes. While the role of Nap1 family proteins in cell cycle regulation and during mitosis has been confirmed, we showed for the first time that a specific member of the Nap1 family, *NAP1L3*, is required for regulation of cell cycle in normal hematopoietic cells and its inhibition causes cell arrest in G0 (Altman and Kellogg 1997, Grande, Lambea et al. 2008). Our data is in line with previous data, which showed that NAP proteins can bind to core histones and physically interact with p300 coactivators to regulate target genes including p53 and E2F (Shikama, Chan et al. 2000). Knockdown of *NAP1L3* in human hematopoietic cells caused an abnormal proportion of mature cells in a xeno-graft mouse model, which might suggest a regulatory role of *NAP1L3* in the epigenetic landscape of HSCs during differentiation to produce all lineages of mature blood cells. Furthermore, *NAP1L3* knockdown decreased the reconstitution and self-renewal capacity of HSCs. Although our RNA-seq data from human HSPCs with *NAP1L3* knockdown did not show significant association with hematopoietic differentiation pathways, we showed that five *HOXA* genes were significantly upregulated upon *NAP1L3* suppression at the mRNA level and two of three investigated at protein level. Since the roles of HOXA genes in self-renewing and differentiation of HSCs are well known, the correlation between NAP1L3 knockdown and upregulation of HOXA genes may give a clue for the role of *NAP1L3* in HSC self-renewal and differentiation (Lebert-Ghali, Fournier et al. 2016).

5 CONCLUDING REMARKS

In order to improve quality of life and increase survival of AML patients, it is vital to increase our knowledge regarding molecular mechanisms that drive the disease and relapse.

New advanced techniques including extensive NGS studies of AML genomes from children and adults have provided a comprehensive map of the genetic and epigenetic abnormalities, as well as the resulting transcriptional changes. Although, all the recurrent mutations in AML have at this time been identified, there are still some key questions needing to be answered, for instance, which mutations can initiate or contribute to the maintenance of AML? Which mutations cause drug resistance and which genes are responsible for relapse?

Therefore, the next big challenge in this field is to identify and functionally characterize genes and pathways with a causative role in the pathogenesis and progression of AML. This will provide better understanding of the underlying mechanisms of AML and thereby help in the development of novel therapeutic strategies. Functional screening including shRNA screening is a promising tool for effective identification of essential genes and pathways in AML. In this thesis, we confirmed the reliability of this technique to uncover crucial novel genes in leukemic cell growth. Moreover, we established a reliable model system and methods including *ex-vivo* expansion of AML and normal hematopoietic cells and intra-femoral transplantation of AML patient cells, to investigate the role of novel genes in AML disease and revealed the functional role of the selected candidates in leukemic cell growth. Hence, our methods might be helpful to shed light on these important questions.

Furthermore, another major reason for the high mortality rate is that few effective drugs have been developed to treat AML. The standard care of treatment, i.e. chemotherapy, has remained mainly unimproved over the past 40 years. During the last decade, numerous studies have been performed to develop new drugs for the treatment of AML but more than 90% of them have failed in the clinical phase. The major problem is that these strategies have not been efficient to completely eliminate AML patient cells and achieve CR or they cause severe side effects on normal cells. Taken together, in AML disease, novel treatment strategies are essentially needed to improve the prognosis for these patients.

One major reason why many new treatments against AML have been failed in clinical trials is due to the intra- and inter-tumor heterogeneity of AML. Moreover, LICs, responsible for the emergence of the disease and relapse after treatment, are very rare in AML cells derived from patients. These are factors that must be considered in any drug discovery research program to provide efficient treatments. In contrast, pharmaceutical companies are typically

limited to using cell lines with poor predictive capabilities, without testing on relevant normal cell based models in pre-clinical trials, which leads to high failure rates in phase I/II clinical trials due to low efficacy and high toxicity profiles of new pharmaceutical compounds.

Since drug development strategies are very time and cost consuming, it might be worth to screen and validate the promising compound candidates first on AML patient samples in particular LICs and normal hematopoietic cells in early stages of drug development then continue with more reliable candidates. Our validation *in vitro* and *in vivo* methods may be a good resource to select more effective targets and new drugs in AML. In conclusion, for future drug development strategies in addition to use advanced techniques, it is important to include patient samples as well as relevant normal cells or tissue models to discover new efficient treatments with curative potential.

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Life is the unique scene of our artistry
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